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Role of T regulatory cells in HIV-2 infection

Thesis submitted to Open University, U.K. in fulfillment of the requirement for the Doctorate of Philosophy in the field of Life Sciences

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Abstract

Infection with the HIV-2 virus can result in progression to acquired immunodeficiency syndrome (AIDS) similar to that seen in HIV-1 infections. However, the majority of HIV-2 infected individuals do not show evidence of disease progression and maintain a functional immune system. It was hypothesized that there was a better balance between effector and regulatory responses in HIV-2 infection that contributed to virus control and immune activation. Furthermore, the role of T regulatory cells was investigated in the development of Immune Reconstitution Syndrome in HIV-1 and HIV-2 patients commencing antiretroviral therapy.

In the present study I first determined the appropriate immunological signature of Tregs in HIV infections. Tregs were defined on the basis of $CD4^+CD127^{lo/-}FOXP3^+$ and were shown to be strongly correlated with $CD4^+CD25^+FOXP3^+$ or $CD4^+FOXP3^+$ cells. However measurement of Tregs using flow cytometry and real time PCR did not correlate. Hence measurement of Tregs from whole blood using flow cytometry was used in subsequent studies.

HIV-2 infected subjects had a significantly lower percentage and absolute number of Tregs compared to HIV-1 infected individuals when matched by CD4 count. In both infections, Treg percentage increased as CD4 T cell count declined while the absolute level was positively correlated with CD4 T cell count. In addition the percentage levels of Tregs positively correlated with the immune activation and viral load in HIV-2 infection only.

The next study measured the level of HIV specific immune responses and immune activation in asymptomatic HIV-1 and HIV-2 patients. HIV-2 infected subjects showed much higher percentage levels of HIV specific CD4 and CD8 T cell and lower percentage levels of activated T cells. Furthermore, it was shown that Tregs in HIV-2 patients were functional as their depletion resulted in increased HIV specific immune responses from CD4 and CD8 T cells.

To assess whether Tregs contributed to the development of Immune Reconstitution Inflammatory Syndrome, HIV-1 and HIV-2 infected patients that were scheduled to commence antiretroviral therapy were recruited and followed up for six months. Longitudinal measurement of Tregs showed no differences in the magnitude or trend of percentage or absolute numbers in patients that developed IRIS compared to those that did not.

Collectively these results suggest that the balance between effector and regulatory responses is maintained in HIV-2 infections which may in turn contribute to the improved prognosis of these patients compared to HIV-1 infections. The IRIS study showed that Tregs did not influence the development of IRIS though further functional characterization of these cells is required.

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Dedicated to my mother Saeeda Zaidi

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List of Abbreviations

AICD	activation induced cell death
AIDS	Acquired immunodeficiency syndrome
APC	allophycocyanin
APC	antigen presenting cell
APT	automatic processing tool
ART	antiretroviral therapy
ATP	Adenosine triphosphate
β2m	β-2 microglobulin
cAMP	cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
CM	central memory
CMV	cytomegalovirus
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
ELIspot	enzyme-linked immunospot
EM	effector memory
FACS	fluorescence-associated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Foxp3/ FOXP3	forkhead/winged helix protein 3
FSC	Forward scatter
Gag	Group specific antigen
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related gene
GUM	Genito-Urinary Medicine
GUSB	beta-D-glucuronidase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen DR1
ICOS	inducible T cell co-stimulator
IDO	indolemine 1,2,3 deoxygenase
IFN-γ	interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IQR	interquartile range
IRIS	immune reconstitution inflammatory syndrome
ITregs	inducible Tregs
LPS	Lipopolysaccharide

LTNP	long term non progressors
LTR	Long terminal repeat
MACS	magnetic bead cell separation
MFI	Median fluorescence intensity
MRC	Medical Research Council
Mtb	Mycobacterium tuberculosis
NK	natural killer
NO	Nitric oxide
nTreg	natural regulatory T cell
PBMC	peripheral blood mononuclear cells
PB	Pacific Blue
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PI	protease inhibitor
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PPD	purified protein derivative (of <i>Mycobacterium tuberculosis</i>)
RBC	Red blood cells
RNA	Ribonucleic acid
RT-PCR	real-time polymerase chain reaction
SEB	Staphylococcus enterotoxin B
SIV	Simian Immunodeficiency virus
SCC	scientific coordinating committee
SCID	Severe combined immunodeficiency
SD	Standard deviation
SSC	Side scatter
STAT	Signal Transducers and Activators of Transcription
TCR	T cell receptor
Th	T helper cell
TGF- β	transforming growth factor beta
TGF- β R	transforming growth factor beta receptor
TNF- α	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor
Tregs	T regulatory cell
VL	viral load

Chapter 1: Introduction

The HIV epidemic continues to spread across the globe despite intense efforts to curb its transmission. The recent UNAIDS report (2010) estimates that there were 33.3 million people who were living with HIV and 2.6 million of these were newly infected. Sub-Saharan Africa continues to bear a disproportionately high burden of disease (Figure 1.1), with 1.8 million new infections in 2009 which represents almost seventy percent of all new infections in this period. Furthermore, of the 1.8 million AIDS related deaths globally, 1.3 million of these were in sub-Saharan Africa. The predominant mode of transmission in sub-Saharan Africa is heterosexual transmission and vertical transmission to breastfed infants. While the number of transmissions in 2009 represents a decline of the peak transmission period in 1999, the scale of the problem remains vast and requires concerted efforts to control transmission of the HIV virus.

The introduction of antiretroviral therapy (ART) in many countries has resulted in a sharp decline in AIDS related deaths. However it is estimated that currently in low income countries only thirty six percent of those needing ART have access to this life saving therapy. Hence, the use of ART alone cannot curtail the HIV epidemic as the worst affected regions are also some of the poorest, which means that the economic cost on the health infrastructure of these countries makes universal ART access unsustainable. The development of an effective vaccine against HIV infections would provide a huge boost to curb its further spread.

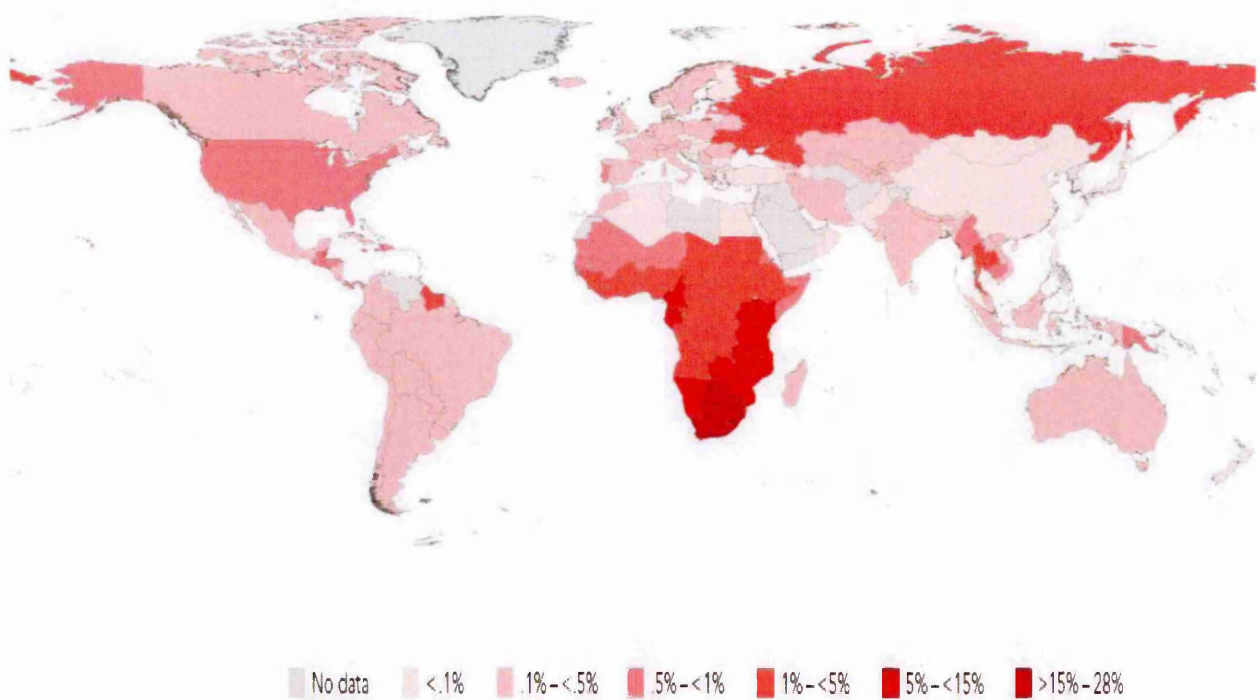


Fig 1.1: Global prevalence of HIV infection 2009 (UNAIDS Report on the Global AIDS epidemic 2010)

HIV-1 was identified almost twenty years ago followed soon after by the identification of a second retrovirus, HIV-2, that can also cause AIDS. The HIV epidemic has been primarily driven by the spread of HIV-1 but to a lesser degree by HIV-2, that is estimated to have infected approximately 1 million cases. HIV-2 was first isolated in 1986 from two AIDS patients from West Africa who repeatedly tested seronegative for HIV-1 (Clavel *et al*, 1986). Both viruses preferentially infect CD4 T cells, integrate into the host genome and upon cellular activation, use the host cell machinery to produce multiple copies which then bud out of the cell and infect new target cells.

The origin of HIV-1 and HIV-2 is thought to be as a result of cross species transmission of Simian Immunodeficiency Viruses (SIV) from Chimpanzees (SIVcpz) and Sooty Mangabeys (SIVsm) respectively. It estimated that such cross species transmissions of SIVsm to

human populations has occurred on at least eight separate occasions giving rise to eight HIV-2 groups. Of these only HIV-2 Group A and B have become endemic, while the other groups represent single person infections (reviewed by Hahn *et al.*, 2000 and Damond *et al.*, 2004).

HIV-2 infections are primarily confined to West Africa, while HIV-1 has spread all over the world (See Section 2.7 for more details). Though the initial study of HIV-2 infections in a sex worker cohort in Senegal suggested that the virus was non-pathogenic, this was found not to be the case for AIDS was described in singly infected HIV-2 patients. The majority of HIV-2 subjects are regarded as long term non-progressors because they maintain high levels of CD4 T lymphocytes accompanied by low or undetectable levels of plasma viremia (Berry *et al.*, 1998). However, a small percentage of HIV-2 patients progress to AIDS, which is indistinguishable from HIV-1 induced immunodeficiency. These patients exhibit a high plasma viral load compared to those with non-progressive HIV-2 as well as a higher degree of systemic immune activation (Lelgiewicz *et al.*, 2009).

It is important to determine the factors that contribute to disease progression in HIV-2 and the underlying mechanisms responsible for slow or non-progression of disease in so many HIV-2 infected subjects. This is important for strategies to design immunotherapeutic and control measures against HIV infection.

Immune response in HIV-2 infection

Many current studies are examining the role of both cellular and humoral immunity in controlling HIV-2 infection. The cellular immune response has been demonstrated to be a pivotal factor in controlling viral replication. Initial studies showed that during the acute phase of infection, a rise in the CD8 T cell response against viral epitopes coincided with a decline in peripheral viral loads (Ogg *et al.*, 1998). Further evidence of the importance of cell mediated immune responses came from experimental SIV infection in monkeys, which showed that ablation of CD8 T cells resulted in a failure to control viral replication during the acute phase of the infection (Schmitz *et al.*, 1999). An early study demonstrated that vigorous CD8 specific T cell response was established against Gag antigens in HIV-2 infection (Gotch *et al.*, 1993). HIV-2 specific CD8 T cells are more polyfunctional compared to those found in HIV-1 specific cells and this polyfunctionality translates to higher levels of cytokine production on a per cell basis (Duvall *et al.*, 2008). The importance of these responses was recently highlighted in a study of HIV-2 infected subjects recruited from a community cohort which showed there was an inverse correlation between CD8 T cell IFN- γ responses targeted towards epitopes in the Gag protein and viral load (Leligdowicz *et al.*, 2007), suggesting that specific cellular immune responses account for viral control in HIV-2 infection.

The quality of CD4 T helper responses, essential for efficient CD8 T cell response, was also found to be strikingly different in HIV-1 and HIV-2 infections (Duvall *et al.*, 2006). Whereas HIV-2 specific CD4 T cell activity showed preserved polyfunctional responses (IL-2 and IFN- γ secretion) in patients with conserved CD4 T cell counts, such responses were absent in HIV-1 patients at a similar stage of disease. Innate immune responses also appeared superior in HIV-2 infection as NK cell activity was also found to be greater in asymptomatic HIV-2 subjects with high CD4 T cell counts than in similar HIV-1 patients.

These differences disappeared in patients showing signs of disease progression (Nuvor *et al.*, 2006). Hence, the immune response directed towards HIV-2 seems effective in controlling HIV-2 replication and may account for differences in plasma viremia between HIV-1 and HIV-2 infections despite a similar proviral load (Berry *et al.*, 1998). However, HIV-2 can result in disease progression similar to that seen in HIV-1 infection and there are few studies to explain the factors that diminish these responses and lead to loss of viral control.

Immune activation in HIV pathogenesis

HIV-1 and HIV-2 infections are characterized by chronic and high levels of activation of the immune system. High immune activation levels have been shown to be predictive of disease progression in both HIV-1 and HIV-2 (Deeks *et al.*, 2004, Giorgi *et al.*, 1999, Hunt *et al.*, 2008, Jaffar *et al.*, 2005). However, whether immune activation levels differ between the two infections remains unclear. One study comparing immune activation levels in HIV-1 and HIV-2 patients adjusted for CD4 counts showed no differences (Sousa *et al.* 2002) despite significantly lower viral loads in HIV-2 patients. In contrast, HIV-2 infected sex-workers in Senegal showed significantly lower levels of activated T cells compared with their HIV-1 infected counterparts (Hanson *et al.*, 2005). A more recent study showed that immune activation consistently correlated with viral load in HIV-2 subjects from a community cohort in Guinea-Bissau (Leligdowicz *et al.*, 2010). This suggests that curbing immune activation is essential for survival of HIV-2 infected individuals. However, there remains no data on how the immune system is able to counter activation in HIV-2 infections so this requires attention.

T regulatory cells in HIV infection

The description of a subset of T cells which behave as regulators of the immune system, was suggested almost four decades ago, but received little support due to the failure to describe their cellular phenotype. While earlier researchers focused on defining suppressive CD8 T cells, the description of a subset of CD4 T cells (aptly termed T regulatory cells) that expressed the α -chain of the IL-2 receptor (CD25), were shown to have suppressive potential in both mice and humans (Sakaguchi *et al.*, 1995; Baecher-Allan *et al.*, 2001). These cells were shown to be a committed lineage of CD4 T cells that were thymically derived and hence came to be known as natural Tregs. The constitutive expression of the transcription factor Forkhead Box Protein 3 (FOXP3) is a feature of natural Tregs and its activity is required to maintain their suppressive phenotype. Natural Tregs modulate both cytokine secretion and proliferative responses from effector cells after stimulation via their T cell receptor using a variety of mechanisms, which are both contact dependent and independent. Whether they assist or hinder the response against HIV-1 is an active topic of research. Earlier studies provided conflicting results on whether their frequencies were elevated or depleted during the course of HIV infection. This was mainly due to the inconsistencies in cellular phenotype, methods used to define their numbers and sampling of heterogeneous population of patients with regards to disease progression. In addition, several studies did not consider the effects of antiretroviral therapy on the Treg population in HIV-1 infected patients. Hence, while some studies have concluded that Tregs play a detrimental role during HIV disease progression by inhibiting HIV specific immune responses (Kinter *et al.*, 2004; Weiss *et al.*, 2004), others have contended that their ability to control immune activation means their activity and presence is required to prevent disease progression (Eggena *et al.*, 2005; Ndlovu *et al.*, 2007) .

Studies characterizing the frequency and function of Tregs in HIV-2 infections alone or in parallel with HIV-1 infections are scarce. This study compared the levels of Tregs in HIV-2 patients in parallel with HIV-1 patients at similar stages of disease progression that were enrolled in the Fajara cohort at the Medical Council Laboratories in The Gambia.

The first aim was to decipher the optimal methods to enumerate Tregs (Chapter 4), and then measure the levels of these cells in HIV-2 infections compared with HIV-1 infected individuals and the relationship with systemic immune activation and viral loads (Chapter 5). The next part of the study was to compare the T cell effector responses in asymptomatic HIV-1 and HIV-2 infections and relate these to immune activation levels (Chapter 6). Finally, the last arm of the study investigated the dynamics of Tregs in HIV-1 and HIV-2 patients after initiation of antiretroviral therapy and their possible involvement in Immune Reconstitution Inflammatory Syndrome (Chapter 7).

Chapter 2: Literature review

2.1 Biology of HIV-1 and HIV-2

HIV-1 was shown to be the causative agent of AIDS by Francoise Barre-Sinoussi's team and was quickly confirmed by other research groups (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984). A second related virus, HIV-2 was later isolated in 1986 from two AIDS patients from West Africa who repeatedly tested seronegative for HIV-1 (Clavel *et al.*, 1986).

HIV-1 and HIV-2 represent the only two retroviruses known to infect humans and cause AIDS. Free virion particles viewed under an electron microscope possess a cone shaped core composed of the viral p24 Gag capsid protein and are approximately 100-120 nm in diameter. The genome of the viruses consists of two identical strands of single stranded RNA approximately 9.8 Kilobases. This encodes three major proteins; Group Specific Antigen (*gag*), Envelope (*Env*) and Polymerase (*pol*), as well as accessory proteins Regulator of Virion (*Rev*), Trans activator of Transcription (*Tat*), Viral Infectivity Factor (*Vif*), Viral Protein R (*Vpr*), Viral Protein U (*Vpu*) and Negative regulatory factor (*Nef*) (Figure 1.2). The HIV-2 viral genome does not contain the *Vpu* gene and instead has the Viral protein X (*Vpx*) gene. The designation of these proteins and their functions are listed in Table 1.1. HIV-1 and HIV-2 share significant homology especially in the *gag* and *pol* regions (60%) but less so for other genes.

The *gag* gene encodes for a large polyprotein designated Pr55^{Gag} that is proteolytically cleaved by the viral protease to yield mature Gag proteins Matrix (MA or p17), Capsid (CA or p26 for HIV-2, p24 for HIV-1), nucleocapsid (NC or p7 for HIV-2, p6 for HIV-1). The *pol* encoded proteins are protease (PR), reverse transcriptase (RT) and integrase (IN), which are transcribed as a large polyprotein precursor designated Pr160^{GagPol} and cleaved by viral PR. The envelope glycoproteins are also synthesized as a polyprotein precursor,

gp160, which is cleaved by cellular proteases to yield the surface and transmembrane glycoproteins gp120 and gp41 respectively.

Gene	Protein(s)	Function
Gag	p24,p17,p7,p7	Capsid protein, structural
Env	gp120,gp41	Envelope surface protein
Pol	p66,p51	Reverse transcriptase
Rev	p19	regulation of viral mRNA expression
Tat	p14	Transactivation
Vif	p23	Assists in proviral DNA synthesis and/or virion assembly
Vpr	p15	Assists in virus replication; Transactivation
Vpu	p16	Helps in virus release by disrupting cell-virion interactions; only present in HIV-1
Nef	p27	Downregulation of MHC molecules on surface of infected cells;
Vpx	p15	Helps in virus entry and infectivity; only present in HIV-2
Protease	p10	posttranslational processing of viral proteins
Integrase	p32	Viral cDNA integration
Tev	p26	Tat/Rev activities

Table 2.1: Gene products of HIV-1 and HIV-2 (adapted from HIV and Pathogenesis of AIDS 3rd Edition)

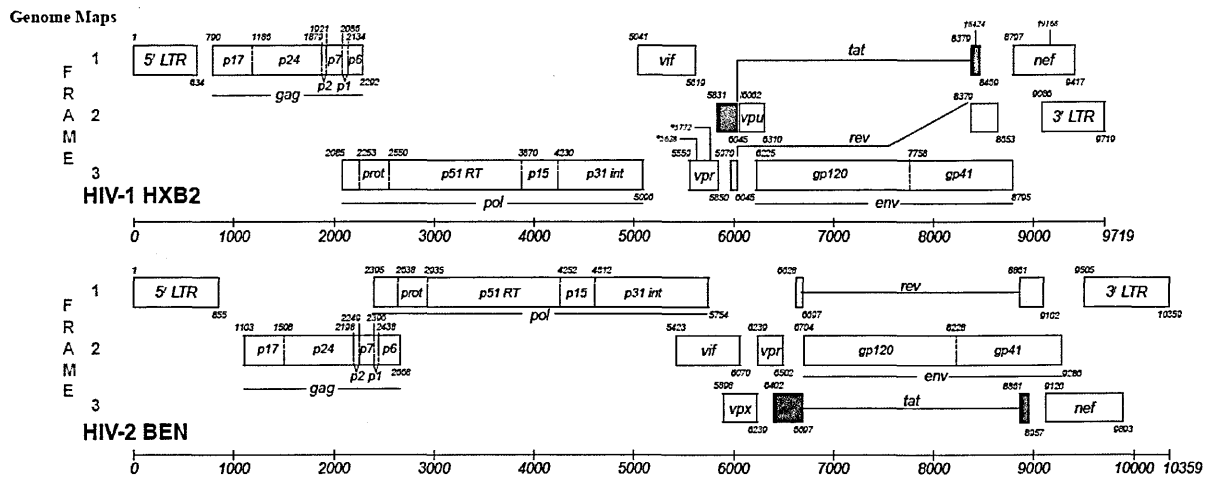


Fig 2.1: Genome organization of HIV-1 and HIV-2 (<http://www.hiv.lanl.gov>)

2.2 Replication of HIV-1 and HIV-2

HIV-1 and HIV-2 infect lymphoid and myeloid cells that express the surface receptor CD4 (Dalglish *et al.*, 1984; Klatzmann *et al* 2004 ; McDougal *et al.*, 1986; Maddon *et al.*, 1986)

The viruses also require the presence of a second co-receptor, which assists viral entry.

The primary co-receptors that are utilized by both HIV-1 and HIV-2 have been identified as C-C chemokine receptor 5 (CCR5) and C-X-C chemokine receptor 4 (CXCR4). However, HIV-2 exhibits higher promiscuity in co-receptor usage and can use CCR1, CCR3, CXCR2, CXCR4, BOB and BONZO *in vitro*, suggesting that it has the potential to target more diverse cell types. (Bron *et al.*, 1997; McKnight *et al.*, 1998; Guillon *et al.*, 1998; Owen *et al.*, 1998; Morner *et al.*, 1999).

Infection of cells requires the interaction of viral gp120 on the surface with the CD4 T cell receptor target cells which triggers a conformational change on gp120 and exposes the binding sites for the chemokine co-receptors. This allows membrane fusion and ultimately release of the viral core into the target cell. The viral core transports the genome to the nucleus using the cellular microtubule network. In order to integrate into

the human genome, the RNA is reverse transcribed to a proviral DNA by the viral enzyme RT. The viral genome is then integrated into the host genome and is flanked by noncoding long terminal repeats (LTR) which regulate the transcription of the integrated proviral genome. The initial steps in integration require the formation of a preintegration complex which consists of the viral DNA molecule, IN, MA, RT, NC as well as host proteins. (Miller *et al.*, 1997, Bukrinsky *et al.*, 1993). The IN enzyme binds to the LTR region and catalyzes the integration of the viral DNA into the host genome (Delelis *et al.*, 2008).

Several studies have shown that the levels of proviral DNA of infected HIV-1 and HIV-2 are similar and suggest that the ability of the two viruses to infect target cells and integrate into the host genomes is comparable (Ariyoshi *et al.*, 1995; Popper *et al.*, 2000; MacNeil *et al.*, 2007).

Once integrated into host chromosomes, the proviral DNA serves as a template that encodes a full complement of structural, regulatory and accessory proteins required for virus replication. The HIV LTR serves as the transcriptional initiator and contains binding sites for host transcription factors NF- κ B and Sp-1. The basal transcriptional activity of the LTR is very low and requires the presence of Tat for significant upregulation of viral RNA production (Pumfery *et al.*, 2003; Romani *et al.*, 2010).

Membrane enclosed HIV particles are generated as the virus hijacks the host cellular machinery to produce multiple copies of itself. The first step requires assembly of infectious viral particles that contain viral Env and the three structural proteins MA, CA and NC that are all generated from the Pr55 polyprotein. The NC protein recruits and packages the viral genome while the CA protein forms the capsid core that encloses this complex.

The HIV virus needs to counter the inhibitory activities of intrinsic host restriction factors whose role it is to block the viral replication cycle. The host restriction factors that have been the most widely studied in the context of HIV-1 include apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), Tripartite motif-containing protein 5 α (TRIM-5 α) and Tetherin. The respective function of these host restriction factors is a) introduction of lethal hypermutations in the virus genome (Sheehy *et al* 2002), b) premature uncoating of the HIV capsid (Stremlau *et al.*, 2006) and c) Prevention of budding of mature viruses from the host cell membrane (Neil *et al.*, 2008).

The accessory proteins of HIV-1 play a major role in overcoming the inhibitory activities of these host restriction factors. The Vif protein targets the APOBEC3G protein for proteosomal degradation, while the Vpu, Env and Vpr proteins have been implicated in overcoming the activity of Tetherin. Also, the high variability seen in the capsid coat protein of the HIV-1 virus may allow escape from the activity of TRIM-5 α .

Once successfully assembled and separated, individual virions separate from the cellular plasma membrane infect new target cells. The number of HIV-1 virions produced per day in the absence of antiretroviral therapy exceeds 10 billion (Perelson *et al.*, 1996), which represents a significant pressure on the immune system and continuous infection of target CD4 T cells. The replication cycle of HIV is summarized in Figure 2.2.

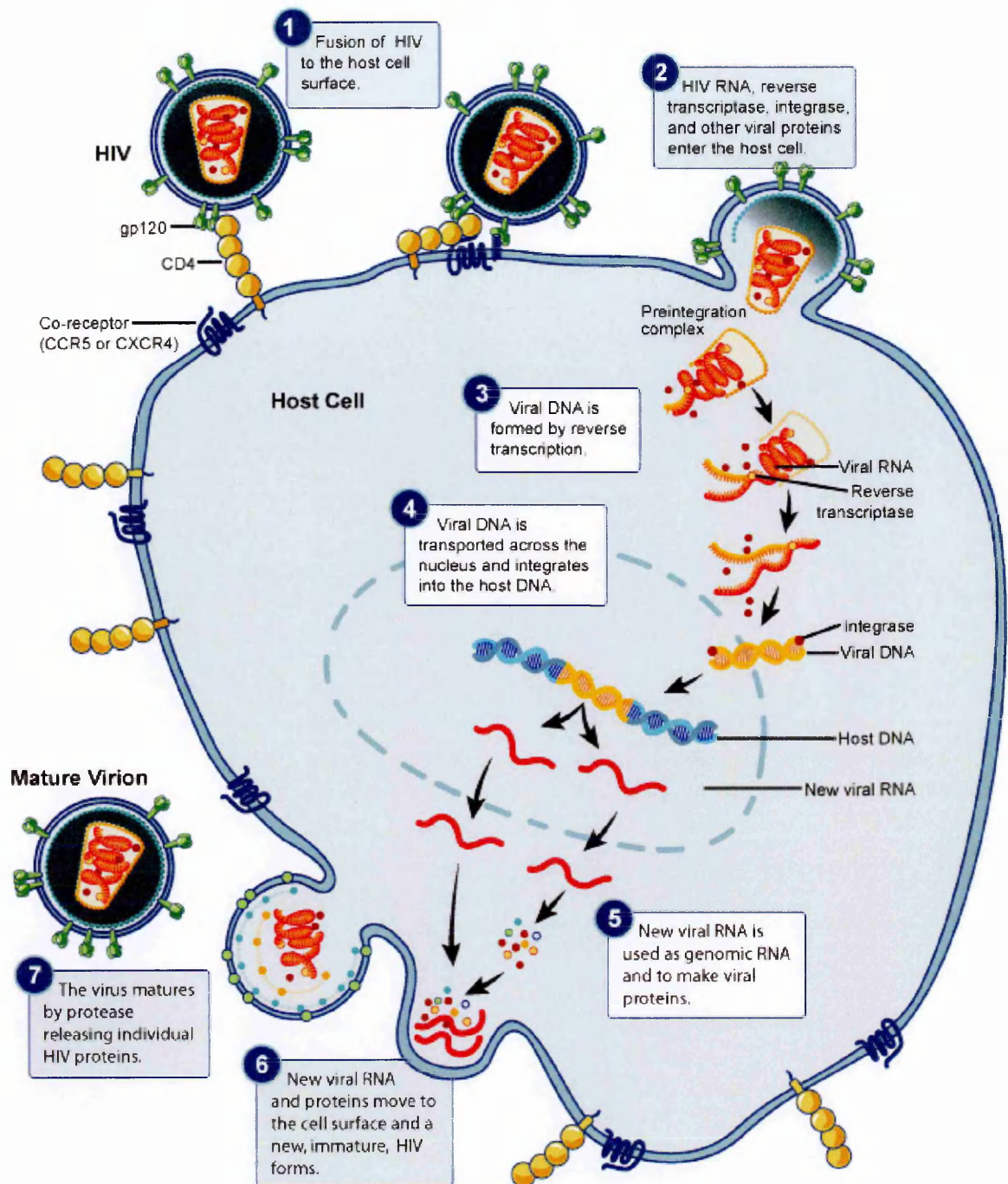


Figure 2.2: Replication cycle of HIV. (www3.niaid.gov/NR)

2.3 Clinical course of HIV infection

HIV-1 infection is characterized by three distinct clinical phases: acute, chronic and the AIDS phase. The acute phase manifests as flu like symptom (Kassutto *et al.*, 2004) and during this time the virus replicates to high levels and leads to massive depletion of CD4⁺ T cells in mucosal tissues (Brenchley *et al.* , 2006). This triggers activation of the host immune system and induction of specific immune responses to the virus leads to lowering of the viral load to a set point. Thus an asymptomatic chronic state is established. In HIV-2 infection, little is known about the early events because of rare detection of incident cases.

The chronic phase is marked by a gradual decline of the CD4⁺ T cells in the periphery which correlates with an increase in markers of immune activation and plasma viral loads. The decline in CD4⁺ T cells can only partially be explained by direct virus effects, since only 1 in 10000 CD4 T cells in the periphery harbour viral proteins or mRNA (Fauci *et al.*, 1986). Immune activation steadily increases during the chronic phase and contributes to loss of CD4⁺ T cells, mediated through combination of direct killing by CD8⁺ T cells, effect of loss of integrity of lymphoid tissues and increased induction of apoptosis.

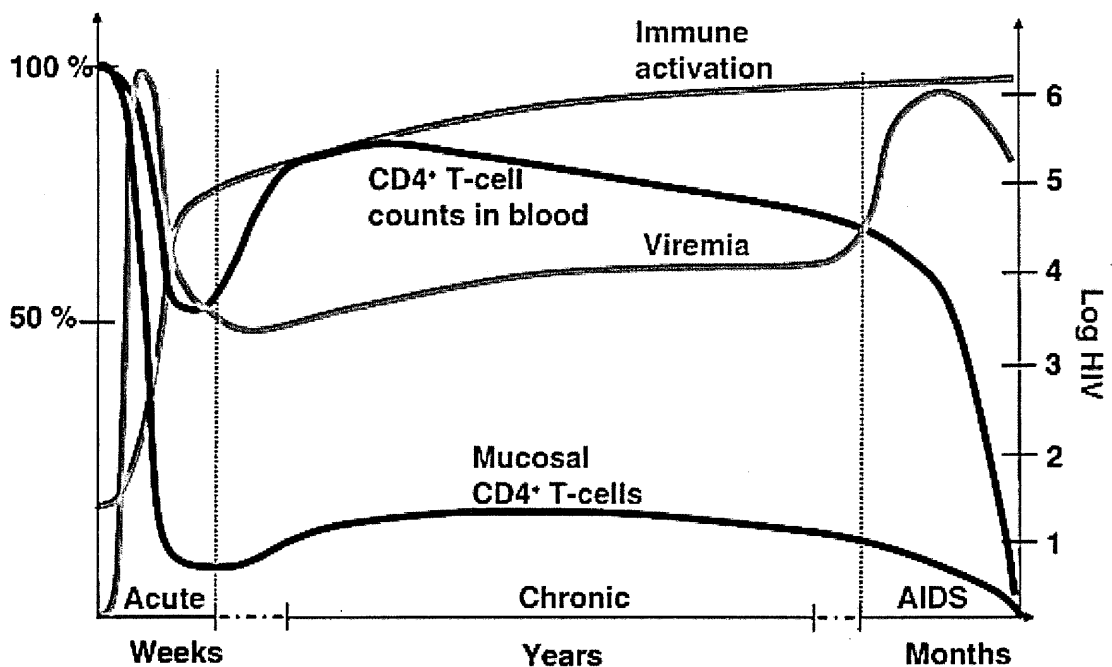


Fig 2.3: Dynamics of peripheral and mucosal CD4 T cell levels, virus replication and immune activation during the long-term course of HIV-1 infection (adapted from Grossman *et al.*, 2006)

The chronic phase in HIV-2 infection is significantly longer than in HIV-1 infection and is characterized by maintenance of high CD4+ T cells accompanied by significantly lower viral loads (Berry *et al.*, 2002; Jaffar *et al.*, 1997; Berry *et al.*, 1998).

In the absence of antiretroviral therapy, the host will eventually progress to the final stage of disease, AIDS. The CD4⁺ T cell levels drop below 350 cells/ μ l and this is accompanied by a dramatic rise in peripheral viremia and immune activation levels. Paradoxically, a profound state of immunosuppression is evident and the host eventually succumbs to opportunistic infections.

2.4 Treatment of HIV infections:

The absence of an effective prophylactic tool has heightened the need for therapeutic measures to reduce HIV induced mortality. In 1987, the first antiretroviral drug, a nucleoside analogue called azidothymidine (AZT), which targets the reverse transcription stage of the virus, was approved for use. Soon after, a number of different nucleoside reverse transcriptase inhibitors (NRTI) were introduced, some more toxic to the host than others. However, it soon became evident that the use of single therapies was ineffective as drug resistant mutations were reported within a year after the introduction of AZT.

A second class of drugs that also targeted the viral RT by blocking the active site of the enzyme (called non-nucleoside reverse transcriptase inhibitors (NNRTI)) were introduced and when used in combination with an NRTI, showed potent suppression of viral replication in HIV-1 patients. The introduction of Protease inhibitors (PI), which target the essential activity of the viral protease, was an important addition to the arsenal of drugs available to treat HIV infection.

A combination of three drugs (termed HAART) became the standard for treatment of HIV-1 patients as it helps circumvent the problem of rapid drug resistance. The current recommendation is for the use of two NRTIs and one NNRTI, since the currently available PI drugs show substantial toxicities. The introduction of HAART resulted in reduction of rate of progression and AIDS related deaths. In the majority of HIV patients commencing therapy, peripheral viremia drops below the limit of detection that is accompanied by substantial recovery of circulating CD4⁺ T cells and reduction in immune activation levels.

Treatment of HIV-2 infections is complicated because of variations within its genome that confer natural resistance to some protease inhibitors (Colson *et al.*, 2004) and reduced sensitivity to NNRTIs (Witvrouw *et al.*, 1999). This severely limits both the first and

second line therapy options in these patients and requires careful monitoring to ascertain that HIV-2 infected patients on HAART respond favourably to therapy.

HAART therapy is a lifelong commitment and cessation of therapy results in a rebound of viral load levels resulting from re-initiation of viral replication from latent reservoirs. HAART therapy is recommended in patients whose CD4⁺ T cell counts have dropped below 350 cells/ μ l, though there remains some debate as to whether this cut-off should be raised. This is especially pertinent since it has been demonstrated that patients commencing HAART at low CD4 T cells counts are more susceptible to developing Immune Reconstitution Inflammatory Syndrome (IRIS) (Valin *et al.*, 2010, Ratnam *et al.*, 2006).

2.5 Immune Reconstitution Inflammatory Syndrome

Immune reconstitution inflammatory syndrome (IRIS, also referred to as immune reconstitution disease (IRD)) occurs in patients commencing ART. This syndrome is characterized by a strong inflammatory reaction and is more common in patients commencing ART therapy with low nadir CD4 T cell count. The majority of patients with onset of IRIS show good response to ART therapy as witnessed by the suppression of viremia to undetectable levels and good recovery of CD4 T cell levels (French *et al.*, 1999). The onset of IRIS typically occurs within the first three months after starting ART and it can affect adherence of patients to the therapy. Most patients resolve their symptoms but it can be fatal in a small number of patients.

IRIS has been broadly classified into paradoxical IRIS (exuberant immune response against residual antigens) or unmasking IRIS (caused by viable pathogens). While the causes of IRIS maybe due to a variety of different pathogens, IRIS associated with *Mycobacterium tuberculosis* (*Mtb*) remains the most widely studied. Other pathogens associated with IRIS

include cryptococci, Kaposi's sarcoma associated herpesvirus, cytomegalovirus (CMV), herpes zoster as well as Hepatitis B (reviewed by Sereti *et al.*, 2010).

The definition of IRIS is still vague since it still relies on clinical exclusion of other factors that maybe causing the symptoms. In *Mtb* associated IRIS it has been demonstrated that higher levels of the T cell cytokine IFN- γ at the time of IRIS compared to non-IRIS cases. A more recent study showed that highly activated memory CD4 T cells produced high levels of IFN- γ after stimulation with *Mtb* derived purified protein derivative (PPD) (Bourgarit *et al.*, 2006). With the increasing access of ART to HIV infected patients there is an urgent need to better define IRIS and understand the factors that may contribute to development of IRIS.

2.6 Microbicides and vaccines for HIV infections

The use of microbicides, and development of prophylactic HIV vaccines, would be important weapons in combating the spread of the disease and significantly reduce HIV associated mortality. However, a number of microbicides that have reached phase 3 testing failed to protect against HIV infection. This could be attributed to a lack of knowledge about the very early events at mucosal surfaces where the viral entry occurs. This is now an active area of research and may lead to development of better microbicides. There is however renewed hope in HIV biomedical prevention research following the success story of the phase IIB efficacy trial of Tenofovir as a microbicide in the CAPRISA004 trial in South Africa. Tenofovir is an adenosine nucleotide analogue and is a potent inhibitor of viral replication. Application of a microbicide gel containing 1% Tenofovir resulted in significantly lower incidence of HIV-1 infections in cases compared to the placebo group (Abdool Karim *et al.*, 2010). Furthermore there was no evidence of development of Tenofovir resistant HIV-1 strains.

The development of HIV preventive vaccine is more elusive. Initial attempts to design a vaccine against HIV-1 infection were focused on inducing neutralizing antibodies against the viral protein gp120, but this failed to protect volunteers from infection (Flynn *et al.*, 2005, Pitisuttithum *et al.*, 2006). The emphasis shifted to designing vaccines that could elicit a strong cytotoxic T cell response, with the hope that this would reduce viral load and hence reduce transmission of the virus. The trial of the Merck vaccine candidate, STEP, designed to elicit HIV specific T cell responses, was stopped early as subjects that received the vaccine had higher infection rates than the control group (McElrath *et al.*, 2008). This failure may have been due to immune activation caused by the adenovirus 5 vector used in the vaccine construct (Buchbinder *et al.*, 2008), though a recent report found no evidence to link adenovirus 5 serostatus to activation within the CD4 T cell

compartment (Hutnick *et al.*, 2009). More encouragingly, the preliminary results of another vaccine trial (RV144) showed a modest reduction in HIV infection rates in vaccinated versus the placebo group. The vaccine efficacy was low (26.4%) and only significant after the removal of 7 subjects from the vaccinated group who were HIV positive at baseline (Rerks-Ngarm *et al.*, 2009). The participants were primed with ALVAC HIV vaccine and boosted with HIV-1 gp120 based AIDSVAX vaccine. These results have galvanized the HIV research community in the belief that a vaccine may yet be possible. However, there remains significant debate about the interpretation of the results of the study, as neither the viremia nor CD4 T cell counts differed in infected patients between the vaccinated or control patients that were infected (Rerks-Ngarm *et al.*, 2009).

These results have highlighted the need to gain a better understanding of the basic biology of HIV infection and the role of immune activation during HIV infection (Barouch *et al.*, 2008). Though the success in designing an effective vaccine has been limited, the information gleaned from these trials have yielded important clues for design of the new generation of vaccine candidates. Indeed, the consensus amongst the HIV research community since these trials has been to advocate a policy of 'back to basics approach' (reviewed by Jose Esparalda *et al.*, 2009), which calls for a better understanding of the virus and the interplay with the host immune system.

There are fewer HIV-1 infected subjects who are long term non-progressors (LTNP). This has made it difficult to explore immune correlates of protection more fully and most initial vaccine designs have relied on animal models of SIV infection. The study of HIV-2 infection, which often results in LTNP of disease, presents an excellent model to investigate the factors that constitute and influence a successful and balanced immune response.

2.7 Epidemiology of HIV-1 and HIV-2 infection in West Africa

The HIV epidemic has been primarily driven by the spread HIV-1 but to a lesser degree by the related lentivirus HIV-2, which accounts for approximately 1 million cases. HIV-2 was first described in West Africa and subsequent cases were detected in India, Mozambique, Angola and Brazil and were attributed to links with the Portuguese who were former colonialists in Guinea Bissau, a country which has the highest HIV-2 prevalence (Poulsen *et al*, 1993). Thus HIV-2 infections are predominantly confined to West Africa. However, unlike the HIV-1 epidemic, which continues to spread globally, the incidence of HIV-2 remains stable or is declining in many of the affected West African countries (van der Loeff *et al.*, 2006). Longitudinal studies conducted in Caio, Guinea Bissau where the prevalence of HIV-2 was between 8-10 % in the 1980s, showed a decline to 4.7% in 2007 in a community cohort. In stark contrast, the HIV-1 prevalence rose sharply in the same community from 0.5% in 1990 to 3.6% in 2007 (van Tienen *et al.*, 2009). Similarly, the prevalence of HIV-2 in a sex worker cohort in Senegal fell from 8% in 1985 to 5.5% in 2003, while HIV-1 prevalence increased from 1% to 13.8% in the same period (Hamel *et al*, 2007). The reasons for the decline in HIV-2 incidence could be primarily due to the lower transmission efficiency. Vertical transmission from HIV-2 infected breastfeeding mothers is only 4% which is six-fold less than that in HIV-1 infected mothers (O' Donovan *et al*, 2000). Sexual transmission is also less efficient as the amount of virus found in genital secretions was significantly lower than in HIV-1 infections (Gottlieb *et al.*, 2006).

HIV-2 infection can result in AIDS similar to that observed in HIV-1 infections. Studies in a clinical cohort in The Gambia (Schim van der Loeff *et al*, 2002) and in two community cohorts in Guinea Bissau did however show that the mortality risk for HIV-2 infected individuals was only two fold greater than uninfected controls (Poulsen *et al.*, 1989,1993), suggesting that HIV-2 was less pathogenic than HIV-1. The clinical features of HIV-2

patients progressing to AIDS is virtually indistinguishable from HIV-1 patients except for the observation that HIV-1 patients were 12.5 times more likely to have Kaposi Sarcoma (Ariyoshi *et al*, 1998). Determining the factors that lead to disease progression in HIV-2 patients would provide valuable information on how a mostly benign infection can lead to immunodeficiency that is as deadly as that seen in HIV-1 infection.

The definitive feature of HIV-2 infection in the majority of cases is characterized by significantly lower levels of circulating virus relative to HIV-1 patients with similar levels of CD4 T cell depletion, even though the amount of integrated virus is similar in both infections (Berry *et al.*, 1998). A longitudinal community-based study of HIV-2 infections in Guinea Bissau showed that low and stable levels of peripheral viremia predicted a normal survival for infected individuals (van der Loeff *et al.*, 2010). In addition, HIV-2 infected individuals that showed little change in their viral load levels maintained high CD4 T cell counts (Berry *et al*, 2002).

Therefore, the lower level of peripheral viremia seen in most HIV-2 infections seems to explain the improved prognosis relative to HIV-1. While HIV-1 replicates to high levels and is readily detectable in the periphery, HIV-2 infected individuals maintain low to undetectable viral loads. This could be a result of the different replication kinetics of the two viruses, as demonstrated *in vitro* (Marchant *et al.*, 2008), or due to better control of virus replication by host factors such as innate restriction factors and the immune response. The immune system, far from being a silent observer, is overly activated in HIV-1 and HIV-2 and it is a key factor in disease pathogenesis of both viruses.

2.8 Immune activation in the pathogenesis of HIV-1 and HIV-2 infection

The hallmark of HIV pathogenesis is chronic aberrant immune activation, which in HIV-1 infection has been found to predict disease progression (Deeks *et al* 2004, Giorgi *et al* 1999, Hunt *et al* 2008 and Liu *et al* 1997). Immune activation is defined by elevated levels of proinflammatory cytokine secretion (TNF- α , IL-6, IL-1 β , MIP-1 α , MIP-1 β and RANTES), polyclonal B cell activation, increased T cell turnover, increased frequencies of cells with an activated phenotype (usually defined by surface expression of CD38 and HLA-DR on T cells) and secretion of β 2-microglobulin and Neopterin. Immune activation is required in the induction of specific immune responses against HIV antigens and control or clearance of an invading pathogen. However, the HIV virus most efficiently infects activated CD4 T cells. Indeed it has been shown that elevated immune activation increases viral replication and infection of new target cells (Grossman *et al*, 1998). Conversely, viral replication also results in increased levels of immune activation as witnessed by elevated levels of CD25 and HLA-DR on bystander CD4 T cells, hence generating increased targets for infection. More specifically, the expression of late activation markers HLA-DR, CD38 or co-expression of both markers on CD4⁺ T cells were associated with increased viral infection and subsequent apoptosis (Biancotto *et al.*, 2008). Chronic immune activation leads to increased T cells turnover and eventual exhaustion of the memory T cell pool and may also lead to damage of thymic tissue required for T cell regeneration (Douek *et al.*, 1998, Schaker *et al.*, 2006).

Multiple factors lead to establishment of immune activation and inflammation in HIV infection. During primary infection, HIV-1 induces a strong CD8⁺ T cell response which persists through to the chronic phase of the infection (Betts *et al.*, 2001). The continuous activation of CD8⁺ T cells could contribute to the loss of infected as well as uninfected CD4⁺ T cells. In addition viral accessory protein *Nef* has been found to induce direct

activation of lymphocytes, macrophages and production of cytokines *in vitro*, which may contribute to the excessive immune activation observed during HIV infection. Furthermore, HIV replication can also cause activation indirectly by depleting CD4⁺ T cells which are required for the control of viruses such as CMV and EBV. Also, the proinflammatory environment generated after HIV-1 infection causes additional bystander activation of concurrent viral infections such as CMV and EBV (Papagno *et al.*, 2004).

The aberrant nature and extent of activation suggests that factors other than the direct viral stimulation are also involved. Indeed recent studies have shown that HIV infection at the mucosal lymphoid tissue leads to massive depletion of CD4⁺ T cells and disruption of the mucosal barriers (Brenchley *et al.*, 2004). This results in increased microbial translocation through the leaky membranes and these microbial products cause activation of the innate immune system (Brenchley *et al.*, 2006). The increased levels bacterial products such as lipopolysaccharide (LPS), flagellin and CpG DNA can activate macrophages and dendritic cells to produce a range of proinflammatory cytokines (IL-6, TNF- α and IL-1 β). Indeed, a recent study showed that immune activation levels correlated with the levels of bacterial DNA in untreated HIV-1 infections, which were lower in treated HIV-1 infections (Jiang *et al.* 2009). Furthermore the levels of soluble CD14 (LPS receptor) have been shown to independently predict mortality in a cohort of HIV-1 infected individuals (Sandler *et al.*, 2011). Taken together these data suggest that microbial translocation is indeed an important determinant of immune activation in HIV infected individuals.

Further evidence that immune activation is a pivotal factor in progression to AIDS has come from the study of non-pathogenic SIV infection in natural hosts such as sooty

mangabeys and African green monkeys. Most infected animals live a normal healthy life in the wild and captivity (Silvestri *et al*, 2007). During the acute stage of infection, immune activation levels are high but subsequently decline even though there is active viral replication as witnessed by high level of viremia ($10^4 - 10^6$ copies/ml) (Broussard *et al* 2001). Thus, the ability of these primates to remain asymptomatic lies in their ability to down regulate immune activation despite lack of viral control.

An intriguing recent study also highlighted the requirement for immune activation at the very early stages of infection. Using an SIV model, Li *et al* (2009) showed that the initial founder infecting population was small but the innate immune response (plasmacytoid DC producing MIP-1 β , MIP-3 α , IL-8, RANTES) attracted more target CD4⁺CCR5⁺ T cells to the site of infections which allowed dissemination of the virus to other tissue sites. Blockade of the initial innate immune response using glycerol monolaurate, a widely used antimicrobial compound, (which specifically inhibits MIP-3 α) protected rhesus macaque females from infection despite repeated exposure to a high dose of SIV (Li *et al.*, 2009). This study further highlights the requirement for immune activation for viral dissemination during infection.

The effect of immune activation in HIV-2 infection has been less well studied. Elevated levels of soluble β_2 microglobulin (β_2 m, a component of the HLA Class 1 molecule) in HIV-2 patients predicted death more accurately than plasma viral load levels (Jaffar, S. *et al.*, 2005). HIV-2 patients at advanced stages of disease show significantly higher levels of immune activation compared to asymptomatic HIV-2 patients. (Michel *et al.*, 2000, Leligdowicz *et al.*, 2008). However, comparison of HIV-1 and HIV-2 patients with comparable CD4 counts showed similar levels of systemic immune activation (Sousa *et al.*, 2002) despite significantly lower viral loads in HIV-2 patients. A separate study

showed lower levels of activated T cells in female sex workers in Senegal infected with HIV-2 compared to HIV-1 subjects but higher than healthy controls (Hanson *et al.*, 2005).

In summary, immune activation plays a pivotal role in progression to AIDS in both HIV-1 and HIV-2, and lower activation levels in HIV-2 may explain the improved outcome in this infection. However, despite the storm of immune activation, an HIV specific response does occur in both infections and has been shown to be important in controlling viral replication. The understanding of the nature of the specific immune responses and how they correlate with protection is still a daunting challenge.

2.9 Host immunity against HIV-1 and HIV-2

Comparisons of immune responses in HIV-1 and HIV-2 infected individuals have helped to identify factors that may contribute to viral replication. Both innate and adaptive immune responses have been shown to contribute to HIV control in the respective infections.

2.9.1 Innate Immunity

The initial response mediated by the host immune response includes production of IFN- α by plasmacytoid dendritic cells (pDC) and proliferation of NK cells, two important components of the innate immune system. As discussed earlier, the contribution of pDC seems to be more detrimental than helpful to the eventual goal of limiting immune activation. In contrast, NK cells are increasingly seen as a key component in recognizing and killing infected cells with the help of killer immunoglobulin receptors that recognize MHC molecules. The function of NK cells, which act directly on infected cells by inducing cytolysis or through the production of chemokine/cytokines, can be severely compromised in patients progressing to AIDS. NK cells from HIV-2 infected individuals displayed higher functionality in comparison to HIV-1 infected subjects with conserved CD4⁺ T cell counts (Nuvor *et al*, 2006), but these differences were absent in patients progressing to AIDS. This suggests that NK cells contribute to the superior control of viral replication observed in HIV-2 infections.

2.9.2. Humoral immunity

The initial adaptive immune response during acute infection is antibody mediated and targeted towards epitopes on the Env, Gag and Pol proteins. Free anti-gp41 IgM antibodies are first seen approximately 13 days after the appearance of the virus in the periphery and undergo class switching to yield IgG and IgA antibodies (Tomaros *et al*, 2008). This is followed in sequential order by antibodies towards gp120, CD4bs and non-

neutralizing gp41 envelope membrane proximal external region (MPER). The appearance of neutralizing antibodies occurs much later (at approximately 13 weeks post infection) and predominantly targets the Env region. (Davis *et al.*, 2009; Moore *et al.*, 2008; Honnen *et al.*, 2007; Rong *et al.*, 2007). However, these antibodies are rare in HIV-1 infected individuals, with barely 1% of chronically infected patients eliciting broadly neutralizing antibodies (Simek *et al.*, 2009; Shen *et al.*, 2009).

Unlike HIV-1, broad neutralizing antibodies are readily detected in HIV-2 patients. Early studies showed the presence of broad neutralizing antibodies in sera from HIV-2 infected subjects which could neutralize heterologous strains but also some HIV-1 strains (Weiss *et al.*, 1988). These results were replicated using sera from HIV-2 patients, which were able to neutralize autologous HIV-2 strains but no such neutralization was observed in sera from HIV-1 patients (Bjorling *et al.*, 1993). A more recent study showed broader neutralizing antibody responses in a large cohort of HIV-2 infected patients compared to HIV-1 patients from the same cohort (Rodriguez *et al.* 2007). While the magnitude was lower, the breadth of neutralizing antibody responses was higher in HIV-2 subjects compared to HIV-1 infected individuals. The fact that the titer is positively correlated to viral load in both infections suggests that neutralizing antibodies elicited are unable to efficiently control viral replication, possibly due to escape mutations. However, the greater breadth of neutralizing antibody responses in HIV-2 infections suggests that this virus may be more susceptible to neutralization.

2.9.3. Cytotoxic CD8 cells

The observation that during the acute phase of HIV-1 infection an increase in cytotoxic CD8⁺ T lymphocytes coincides with a decline in viral load levels in the blood, provided evidence that these cells played a pivotal role in viral control. Further proof that CD8⁺ T

cells control virus replication *in vivo* was evident in the SIV model in non-human primates, whereby depletion of CD8⁺ T cells resulted in failure to control the virus both in early infection and during the chronic phase (Schmitz *et al.*, 1999). A seminal study by Betts *et al.* (2006) further demonstrated that the quality of the CD8 T cell response was an important correlate of protection. Comparison of HIV specific CD8 T cell responses in HIV-1 infected progressors and non-progressors showed higher degree of polyfunctionality in the latter group (as measured by the simultaneous production of cytokines IL-2, IFN- γ , MIP-1 β , CD107 mobilization and proliferation).

Similar to HIV-1, vigorous anti-Gag CD8⁺ T cell responses are evident in HIV-2 infected individuals (Gotch *et al.*, 1993). Surprisingly, comparison of the magnitude of IFN- γ release by CD8⁺ T cells in response to Gag in HIV-1 and HIV-2 patients showed no significant differences (Jaye *et al.*, 2004). However, studies comparing T cell receptor usage in the two types of infection showed that the plasticity of immune responses was superior in HIV-2 judged by a broader polyclonal CD8⁺ T cell repertoire (Lopes *et al.*, 2003). This suggested that the ability of HIV-2 to escape the immune response may be limited in comparison to HIV-1 as CD8⁺ T cells from HIV-1 infected patients had a much narrower TCR usage. In addition, HIV-2 specific CD8⁺ T cells have been shown to exhibit higher polyfunctionality which translated to higher levels of cytokine production on a per cell basis (Duvall *et al.*, 2008). The importance of these responses was recently highlighted in a study of a community based study HIV-2 infected subjects which showed an inverse correlation between T cell IFN- γ responses targeted towards epitopes in the Gag protein and viral load (Leligdowicz *et al.*, 2007), suggesting that Gag specific cellular immune responses accounts for viral control in HIV-2 infection.

2.9.4. CD4 T-cell response

CD4⁺ T cell specific responses, especially towards the Gag region, do occur in both HIV-1 and HIV-2 infections, but the magnitude is lower compared to CD8⁺ T cells (Duvall *et al.*, 2007). The quantity and quality of CD4⁺ T helper responses is strikingly different in HIV-1 and HIV-2 infections (Duvall *et al.*, 2006). Whereas HIV-2 specific CD4⁺ T cell activity showed preserved polyfunctional responses (IL-2 , IFN- γ and proliferation) in patients with conserved CD4⁺ T cell counts, such responses were absent in HIV-1 patients.

However, in HIV-1 and HIV-2 infections both CD4⁺ and CD8⁺ effector T cell responses decline as disease progression occurs. In addition to immune escape mutations, other factors that contribute to the diminishing responses include expression of immune inhibitory molecules such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programme death-1 (PD-1). The level of expression of these markers on CD4⁺ and CD8⁺ T cells correlates with disease progression in HIV-1 infections (Kaufmann *et al.*, 2007, Trautmann *et al* 2006, Day *et al* 2006) but as yet there remain no similar studies in HIV-2 infections.

The host immune response requires a delicate balancing act whereby it must contain excessive immune activation while allowing specific anti-HIV responses to successfully control viral replication. As the majority of HIV-2 infections progress slowly and immune activation is reduced, it is possible that the balance between regulatory and effector responses is better compared to HIV-1 infections. A subset of CD4⁺ T cells that curtail exuberant immune activation, termed regulatory T cells (Tregs), maybe key players in mediating this balance.

2.10 T regulatory cells

The concept that the immune system has a dedicated mechanism to prevent immunopathology had been suggested as early as 1970 (Gershon and Kondo 1970, Gershon 1975). The idea of suppressor cells was temporarily abandoned due to a failure to find specific markers that could be used to define these cells. The discovery of CD4⁺ T cells that expressed CD25 (the high affinity chain of the IL-2 receptor) and exhibited immunoregulatory properties in mice (Sakaguchi *et al.*, 1995) reignited interest in suppressor cells, which were renamed as T regulatory cells. A similar population was soon described in humans but unlike mice, only CD4⁺ T cells that expressed the highest amount of CD25 (CD25^{hi}) showed suppressive function (Baecher-Allan *et al.* 2001).

A major breakthrough in identifying a specific marker for Tregs was made when the forkhead winged helix protein 3 (FoxP3, denoted FOXP3 in humans) was discovered as the defective gene in the mouse strain Scurfy (Brunkow *et al.*, 2001). FOXP3 is encoded on the X-chromosome and belongs to the forkhead/winged helix family of transcriptional regulators which are characterized by the presence of the FKH domain required to bind target DNA sequences (reviewed by Ziegeler *et al.*, 2006). FoxP3 expression was shown to be restricted to CD4⁺CD25⁺ T cells and CD4⁺CD8⁻CD25⁺ thymocytes in normal mice. Furthermore, ectopic expression of FoxP3 in CD4⁺CD25⁻ cells conferred regulatory properties to these cells (Fontenot *et al.*, Hori *et al.*, 2003, Khattry *et al.*, 2003). Scurfy mice suffer from an aggressive CD4⁺ and CD8⁺ T cell-mediated autoimmune disease with multi-organ inflammation and die at the age of 3 to 5 weeks. Conversely, depletion of FoxP3⁺ cells in young male mice resulted in a Scurfy like phenotype (Fontenot *et al.* 2003). A similar condition is seen in humans, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which results in aggressive autoimmunity and death

within the first two years of life. This has also been linked to mutations in the human FOXP3 gene (Gambineri *et al.*, 2003; Bennett *et al.*, 2001).

FOXP3 is essential for the function of Tregs and its expression is required to maintain the suppressive phenotype. Loss of FOXP3, or diminished expression of this gene, results in acquisition of effector functions characterized by the production of cytokines such as IL-2, IFN- γ , IL-17 and IL-4 (Wan and Flavell 2007; Williams and Rudensky 2007). In addition, *in vitro* transfection of FOXP3 into human CD4⁺CD25⁻ cells is sufficient to impart suppressive function on these cells. Taken together these data show that FOXP3 is essential for the Treg phenotype.

2.11 Differentiation of Tregs

Commitment to the Treg lineage has been associated with the induction of FOXP3 in thymocytes during their development. Recent data suggests that Treg cell differentiation occurs in two steps in the thymus. The first step is dependent on high affinity interactions between the T cell receptor (TCR) and its cognate antigen, which results in expression of CD25^{hi} FoxP3⁻ single positive CD4 thymocytes. The second step requires the presence of IL-2, which is required to convert the intermediate population to mature CD25⁺FoxP3⁺ cells (Lio *et al.*, 2008; Burchill *et al.*, 2008), in a STAT5 dependent manner. In addition to TCR engagement and the requirement for IL-2, CD28 co-stimulation seems to play an important role in differentiation of thymic derived Tregs as mice deficient in CD28 or CD80/CD86 have markedly decreased levels of circulating Tregs (Salomon *et al.*, 2000; Tai *et al.*, 2005).

The generation of Tregs can also occur in the periphery. Both human (Fantini *et al.*, 2004) and murine (Betteli *et al* 2006, Chen *et al* 2003) naïve T cells have been shown to express FOXP3 and acquire suppressive activity after TCR stimulation in the presence of TGF- β

and IL-2 (Chen *et al.*, 2003). The gut associated lymphoid tissue appears to be a preferential site for peripheral induction of FOXP3⁺ Tregs (Coombes *et al.*, 2007; Mucida *et al.* 2005,2007 ; Sun *et al.* 2007) and DCs from the small intestine and mesenteric lymph nodes appear to be better inducers of FOXP3 in naïve T cells than splenic DCs in the presence of exogenous TGF- β . A different theory suggests that conversion of proliferating memory CD4⁺ T cells to Tregs is a predominant mechanism of induction of Tregs (Vukmanovic-Stejić *et al.*, 2006) in response to foreign antigens. Using deuterium labeling, the authors highlighted parallel increases in effector and Treg populations that utilized the same TCR V β families. A more recent study showed that stimulation of human PBMCs with bacterial superantigens resulted in a dose dependant induction of FOXP3 in CD4⁺CD25⁻ cells (Taylor *et al.*, 2010).

Until recently, it was not possible to distinguish thymus derived Tregs from peripherally induced Tregs. Studies exploring the demethylation status of FOXP3 identified key differences in natural and induced Tregs. While natural Tregs showed complete demethylation of a specific locus named as Treg cell specific demethylated region (TSDR) found on exon 1 of the FOXP3 gene, this locus was only partially demethylated in induced Tregs (Floess *et al.*, 2007; Huehn *et al.*, 2009). Furthermore, thymically derived Tregs display high expression of the transcription factor Helios which is absent in peripherally induced Tregs (Thornton *et al.*, 2010).

2.12 Treg phenotype

The phenotype of mature Tregs remains a contentious issue. Tregs constitutively express high levels of FOXP3 and these cells display low or negative surface expression of IL-7R α (Seddiki *et al.*, 2006). Although CD25 was the initial marker used to define Tregs, it has become apparent that FOXP3 expression does occur in CD25⁻ cells as well (Finney *et al.*,

2010), leading to suggestion that CD25 maybe a marker of activation on Tregs (Finney *et al* 2009). Tregs also express high levels of cytotoxic T lymphocyte antigen 4 (CTLA4), CD39, CD73, though none of these markers are exclusive to Treg cells. To date the most accurate description of Tregs remains $CD4^+CD127^{lo/-}FOXP3^+$ and is now widely used to enumerate these cells.

2.13 Mechanism of Treg action

Tregs utilize multiple mechanisms to suppress effector responses such as the secretion of IL-2, IFN- γ and proliferation. The intriguing question which was posed early, but as yet not conclusively answered, was whether Tregs require direct contact with target cells to mediate their suppressive function. While most of the *in vitro* data suggested that the predominant mechanism of suppression was contact-dependent, *in vivo* results from mouse models indicate that the mode of suppression could be cytokine mediated. However, such data is difficult to obtain in human studies and has been complicated by the failure to identify a soluble mediator emanating from Tregs.

Evidence for the requirement for direct cell contact by Tregs to mediate their suppressive capacity has come from Transwell experiments. Physical separation of Tregs and effector cells results in enhanced proliferative and cytokine responses in the latter subset after TCR stimulation, suggesting that cell contact is required by Tregs to exert control over effector functions. Do Tregs exert their suppressive activity by acting directly on effector cells or on antigen presenting cells? *In vivo* imaging experiments in mice have highlighted the fact that Tregs swarm around DCs and hinder prolonged contact between DC's and naïve T cells, suggesting abortive priming of effector cells (Tang *et al* 2006; Tadokoro *et al* 2006). The precise ligands that mediate Treg-DC contact are still not clear but it appears that the co-stimulatory molecule CTLA-4 is a major contributor to Treg function. CTLA-4, a

member of the CD28 superfamily, is a co-stimulatory molecule which is constitutively expressed on Tregs. In the absence of functional CTLA-4 or its blockade using antibodies resulted in reduced suppression of effector T cells by DCs (Oderup *et al.*, 2006; Serra *et al.*, 2003). CTLA-4 ligation of co-stimulatory molecules CD80 or CD86 also resulted in production of indoleamine 2,3, dioxygenase (IDO), which catabolises tryptophan to yield proapoptotic metabolites that are potent suppressors of effector T cells (Fallarino *et al* 2003 ; Mellor *et al* 2004). Other studies have shown that Tregs can down regulate CD80 and CD86 expression *in vitro* on DC's which would reduce their ability to prime effector T cells (Cedeborn *et al.*, 2000). However, a more recent study showed that Tregs were more mobile than effector T cells after TCR stimulation and better at aggregating around DCs, which was independent of CTLA-4 but required the presence of Leukocyte function-associated antigen -1 (LFA-1). CTLA-4 was however required for downregulation of CD80 and CD86 on DCs in agreement with other studies. (Onishi *et al* 2008). Several other studies have suggested that Treg mediate modulation of the maturation and function of DCs (Kryczek *et al* 2006; Lewkowich *et al.*, 2005., Houot *et al.*, 2006., Misra *et al.*, 2004)

More recently another mechanism of suppression has been described which is dependent on the activities of two ectoenzymes CD39 and CD73 (Deaglio *et al.*, 2007). The expression of both these enzymes has been shown to be upregulated on Tregs. The concordant expression of these two enzymes results in the pericellular generation of adenosine, which is a potent suppressor of proliferation of effector T cells, as well as encouraging the induction of adaptive Tregs. Adenosine has a half-life of 10 seconds which suggests that there needs to be close proximity between Tregs and effector cells for the effects of this molecule to be maximized. The generation and subsequent transfer of cyclic AMP from Tregs to effector cells via gap junctions has also been highlighted as a

potential mechanism of Treg suppression (Bopp *et al.*, 2007). These mechanisms need further investigation and could yield potential mechanisms to that can be used to modulate Treg activity.

2.14 Tregs and infectious diseases

The outcome of an infection is usually dictated by action of the host immune response. While some pathogens are cleared, others persist despite the induction of a robust immune response. The pioneering study of the role of Tregs in infectious diseases was one that showed Treg abrogate Th1 responses required for clearance of *Leishmania* from sites of primary infection in a mouse model (Belkaid *et al.*, 2002). A small number of parasites persist in tissue lesions, which was found to be dependent on IL-10 emanating from CD4⁺CD25⁺ Tregs. Parasite persistence was required for maintenance of protective immunity but at the same time T cell mediated immunopathology needs to be avoided. The realization that Tregs are important modulators of the host immune response during infection led to a whole plethora of studies investigating their role in a variety of infectious diseases.

In malaria, Tregs induced after sporozoite challenge correlated with pro-inflammatory responses and higher parasite loads (Walther *et al.*, 2005). In a follow-up to this study, Finney and colleagues demonstrated that Tregs levels were higher among individuals living in a rural village in Gambia with high malaria transmission compared to an urban area with negligible malaria transmission (Finney *et al.*, 2009). This study suggested that Tregs were essential in maintaining immune homeostasis in environments with frequent persistent antigenic exposure. Also, Treg activity during acute *Plasmodium falciparum* malaria dictated subsequent malaria specific memory responses in children (Walther *et al.*, 2009). Similarly, the levels of Tregs have been linked to clinical outcome in West Nile

virus infections, which leads to symptomatic disease in approximately 20% of infected subjects: a longitudinal study of Tregs in infected patients showed that asymptomatic patients had significantly lower levels of Tregs compared to patients who developed clinical symptoms of disease (Lanteri *et al.*, 2009). Collectively these studies suggest that induction of Tregs early after an infection has a bearing on the subsequent clinical outcome of acute infections.

During most chronic infections Tregs levels are elevated and accumulate at the sites of infection. During *Mtb* infections, Treg cell numbers are increased both in the blood and sites of infection and the frequency of FOXP3⁺ cells inversely correlates with *Mtb*- specific immunity (Chen *et al.*, 2007, Qin *et al.*, 2008, Roberts *et al.*, 2007, Hougardy *et al.*, 2007). Using a mouse model of *Mtb* infection, a recent study suggested that increased levels of *Mtb* specific Tregs may be detrimental and contribute to delayed priming and trafficking of effector cells to the site of infection, thus allowing the bacteria to persist (Shafiani *et al.*, 2010). Similarly, FOXP3⁺ cells accumulate in the conjunctiva of patients infected with *Chlamydia trachomatis* (Faal *et al.*, 2006). Chronic HBV infection is characterized by an elevated frequency of FOXP3⁺ cells in the periphery as well as in the liver and correlates with viral load (Peng *et al.*, 2008, Stoop *et al.*, 2007). Expansion of HBV specific Tregs have been associated with liver pathology evident during infection (Xu *et al.*, 2006). In HCV infections Treg levels are raised in chronic infection and accumulate in the liver (Smyk-Pearson *et al.*, 2008; Ward *et al.*, 2007). Furthermore, these cells were shown to exhibit high suppressive ability as depletion of Tregs *in vitro* from chronically infected HCV patients' results in increased CD8⁺ T cell responses.

These studies highlight the significant role played by Tregs in the pathogenesis of various infectious diseases and may in the future serve as targets for intervention to treat these diseases.

2.15 Tregs in HIV: Friend or foe?

The role of Tregs in HIV infection continues to generate strong interest and controversy due to conflicting data about their potential role in disease pathogenesis. The chronic stage of HIV-1 infection is characterized by the progressive loss of CD4⁺ T cells accompanied by persistently high levels of immune activation and immune dysfunction. Tregs act to curtail excessive immune activation and so in the context of HIV-1, the debate as to whether Tregs help or hinder control of HIV infections rages.

Many of the early studies investigating the role of these cells in HIV infection focused on whether Tregs were responsible for diminution of cell mediated HIV-1 specific responses. Since conjugated antibodies towards FOXP3 were not available, most of these studies used CD25 expression on CD4⁺ T cells or mRNA levels of *FOXP3* to define Tregs. *In vitro* depletion of CD25⁺ cells from HIV-1 positive individuals resulted in increased anti-HIV specific immune responses (Kinter *et al.*, 2004 , Andersson *et al.*, 2005; Weiss *et al.*, 2004; Aandahl *et al.*, 2004) , suggesting that Tregs played a detrimental role in HIV-1 disease by reducing HIV-specific immune responses. In contrast, other reports suggested that failure to control the excessive levels of immune activation seen in HIV-1 infection was a direct result of reduced Treg frequencies or impaired suppressive ability of these cells (Oswald-Richter *et al.*, 2004; Apoil *et al.*, 2005). However, these early studies were clouded by differences in the phenotype used to define Tregs, differing methodologies used to measure Tregs and sampling of HIV-1 patients at different disease stages.

More recent studies, which included FOXP3 as a signature for Tregs, have shown that the frequencies of Tregs (expressed as a percentage of total CD4+ T cells) are increased in the periphery (Tsunemi *et al*, 2005; Montes *et al.*, 2006, Rallon *et al.* 2009) and in mucosal tissues (Epple *et al.*, 2006) during the chronic stage of HIV-1 infection and inhibited HIV specific immune responses. Moreover, other studies showed that Tregs accumulation in lymphoid tissue correlated with viral replication in HIV-1 infection and with progression to AIDS (Nilsson *et al*, 2006). Collectively these studies suggest that Tregs inhibit HIV specific responses and allow viral persistence.

HIV replication causes an incremental increase in immune activation through direct or indirect effects and Treg mediated control of such activation would be required. However, Treg activity also results in diminution of HIV specific immune responses which may assist viral persistence. Furthermore, Tregs are targets of HIV infection and can be induced in response to increased levels of activation. Defining the precise role of Tregs in the pathogenesis of HIV-2 infections would certainly assist in understanding the factors that contribute to successful viral control.

2.16 Tregs in SIV infection

The study of Tregs in pathogenic and non-pathogenic models of SIV infection has yielded important clues about the role these cells play in controlling generalized immune activation and SIV specific immune responses. The distinguishing feature of non-pathogenic SIV infection is the lack of persistent immune activation even though high levels of viral replication occurs in both models (Silvestri *et al.*, 2003). A strong anti-inflammatory profile, which comprised of an early up regulation of TFG- β and IL-10 in African green monkeys, coincided with an increase in Tregs and was associated with protection from AIDS (Kornfeld *et al.*, 2005). Furthermore, depletion of Tregs was

observed in the GALT of SIV infected macaques (Chase *et al*, 2007) which correlated with loss of suppressive activity of CD4+ T cell from these animals. The dynamics of Tregs in acute SIV infections have shown its early upregulation in both pathogenic and non-pathogenic models but these cells are transiently lost in the pathogenic model. Another important subset of CD4+ T cells capable of producing IL-17 (aptly referred to as Th17 cells) were also maintained in non-pathogenic SIV infection, and the ratio of Th17/FoxP3 positive cells was inversely proportional to CD8+ T cell activation levels in blood, lymph nodes and mucosal tissue (Favre *et al.*, 2009). A more recent study linked the suppressive capacity of Tregs to maintenance of high CD4 T cell counts in experimentally infected Macaques (Karlsson *et al.*, 2010). Therefore, in the context of non pathogenic SIV infection, Tregs appear to play a beneficial role during the acute stage of infection by controlling excessive immune activation levels which helps prevent progression to AIDS.

As yet there are few comparative studies of Treg frequencies or function in HIV-2 infections. HIV-1 and HIV-2 represent infections with similar viruses but distinctly different outcomes. We present a comparative analysis of the phenotype, frequency and function of Tregs in HIV-1 and HIV-2 infections in a West African Clinical cohort.

2.17 Rationale of the study

Studies of the role of Tregs in progressive versus non-progressive disease in humans are scarce. Although, studies in SIV infection models provide useful insight into pathogenesis of AIDS like illness, there are substantial differences with the HIV infection model. HIV-2 remains the only other lentivirus known to infect humans and can cause AIDS. The majority of HIV-2 infected patients behave as long term non-progressors characterized by well functioning immune responses accompanied by lower levels of systemic immune activation. We hypothesized that the balance between effector and regulatory responses is better preserved in HIV-2 compared to HIV-1 infections that explains the improved prognosis in HIV-2.

The objectives of the study were:

- 1) Enumerate Treg frequencies in chronic HIV-1 and HIV-2 infected individuals (progressors and non-progressors)
- 2) Relate Treg frequencies to immune activation, viral load levels and CD4+ T cells counts in HIV-1 and HIV-2
- 3) Determine the balance between effector and regulatory responses during asymptomatic HIV-1 and HIV-2 infections
- 4) Explore the effects of HAART on Treg frequencies and explore their potential involvement in the development of immune reconstitution syndrome

Chapter 3: General Methods

3.1 Patients

The studies described in the subsequent chapters of this thesis were conducted at the MRC Laboratories in The Gambia. The Gambia is located on the West Coast of Africa and has one of the smallest land masses on continental Africa. The Gambia has a population of 1.6 million and the predominant ethnic tribes are Mandinka (42%), Fula (18%), Wolof (16%) and Jola (10%). The other ethnic groups include the Manjago, Bambara and Krio Aku Marabout. The majority of Gambians practise Islam (90%) as their religion while Christianity and traditional religions are also practised by approximately 10% of the population.

The MRC Laboratories in The Gambia has been active since its inception in 1947. The MRC runs a specialized Genito-Urinary Medicine (GUM) clinic which acts as a referral centre for HIV infected patients. Patients visiting this clinic were enrolled into the Fajara cohort after giving informed consent. These patients typically visited the clinic 3-6 times a year and provided a sample of blood for viral load and CD4 monitoring. At the end of 2008, there were 3995 patients registered at the clinic of which approximately 2200 patients were known to be alive. HIV-2 infected patients constituted twenty seven percent of these patients with a further seven percent were dually infected with both HIV-1 and HIV-2.

HIV-1 and HIV-2 patients enrolled into the MRC Fajara cohort were consented and enrolled in to the study. A total of eighty three HIV-1 and 72 HIV-2 patients who were antiretroviral therapy naive, >18 years of age and did not present with any concurrent infections such as tuberculosis and malaria were recruited for the studies. Any patients that were dually infected with both HIV-1 and HIV-2 were excluded from the study.

Another eighty patients were recruited for the IRIS Study at the time they were to commence antiretroviral therapy and sampled at time 0,1,2,3 and 6 months after commencement of therapy. These patients received counseling from experienced staff at the MRC before commencement of therapy and were encouraged to visit the GUM clinic if they experienced any adverse events. Viral load and CD4 T cell counts were measured for all patients for each individual visit to the GUM clinic.

These studies were approved by the Gambia Government/MRC Ethical Committee (see Appendix).

3.2 HIV Diagnosis and viral load testing

HIV screening was performed using the Murex ICE HIV-1.2.0 capture enzyme immunoassay (Murex Diagnostics). Patient samples that displayed a positive result were further tested using a Hexagon (Human GmbH, Germany) line assay which measures antibodies against viral proteins gp41, p24 and gp36 in plasma to discriminate between HIV-1 and HIV-2 patients. In the event of an indeterminate result, plasma samples were further tested using the Pepti-Lav kit (Sanofi Diagnostics, Pastuer).

Plasma viral load was quantified by an in-house reverse transcriptase assay as previously described (Berry *et al.*, 1998; Alabi *et al.*, 2007). Undetectable viral load was assigned an arbitrary value of 100 copies/ml and the upper detection limit of the assay was 1000000 copies/ml.

3.3 CD4 T cell counts

Enumeration of CD4 levels (CD4% of total CD3 T cells) was done using BD MultiTest reagents and MultiSet software (BD Biosciences, USA). Briefly, 50µl of Whole blood was incubated with 10ul of the Multitest (BD Biosciences, UK) antibody (anti-CD4, anti-CD8, anti-CD3, and anti-CD45) cocktail. The cells were then lysed with 1X FACS Lysis solution

(BD Biosciences, UK) and acquired on a 4-color FACS Calibur using the Multiset software. Whole blood counts and lymphocyte percent were measured using a Medonic haemoanalyzer (Clinical Diagnostic Solutions Inc, USA), which was used to calculate the absolute numbers of CD4⁺, CD8⁺ and CD3⁺ T cells per µl of whole blood.

3.4 Whole Blood FACS Staining for cell surface and intracellular markers

Whole blood FACS staining was performed on freshly isolated venous blood and 150µl was used per tube. This amount of blood was also used to stain for the individual conjugated CD8 antibodies that were required for adjusting compensation values on the flow cytometers.

A cocktail of surface antibodies was prepared (CD4-PerCP, CD25-FITC, and CD127-PE) and added directly to 150 µl of whole blood. After a 30 min incubation at room temperature in the dark, the cells were washed with FACS Buffer (PBS containing 2% BSA, 0.2 % EDTA) to remove any unbound antibody. The cells were then fixed and permeabilised using 500µl of Fix:Perm buffer (Ebiosciences, San Diego, USA) and incubated for 30min at 4°C to facilitate detection of intracellular markers. The cells were then washed with FACS Buffer and then with Perm Buffer (Ebiosciences, San Diego), and resuspended in 50 µl of Perm Buffer containing 2% Rat Serum. After 15 min of incubation at 4°C conjugated antibodies for intracellular marker (FOXP3-APC) were added. The cells were then incubated for 30min at 4°C, washed with Perm Buffer and resuspended in 200µl of PBS. The cells were collected on a four-color FACSCalibur (BD) instrument. Data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

3.5 β 2-microglobulin measurement

140 μ l of Plasma was used to determine the levels of soluble β 2- microglobulin (β 2m), which is a component of the major histocompatibility complex (MHC) class 1 found on all nucleated cells Soluble levels of β 2m have been shown to be a good marker of immune activation in HIV infected patients and reliably predict progression to disease (Jaffar *et al* 2005).

Undiluted plasma from HIV-1 and HIV-2 patients was used to measure β 2m levels using the automated AxSYM β -2 microglobulin assay (Abbot, USA). This assay relies on anti β 2m antibody coated microparticles that bind soluble β 2m in the plasma sample forming an antigen-antibody complex. Anti β 2m: Alkaline Phosphatase conjugate is first added and binds to the antigen-antibody complex. After washing any unbound conjugate the substrate 4-Methylumbelliferyl Phosphate is then added and the resulting fluorescent product is measured against a standard curve generated from known standards provided by the manufacturer. The lower limit of detection was 200 μ g/L, while the upper limit of detection was 4000 μ g/L. Samples, in which β 2m levels were above the upper limit, were retested using the automated dilution protocol (1 in 20 dilution).

3.6 Isolation of PBMC

Up to 15ml of venous blood were collected in a heparinised tube. The samples were centrifuged for 5 minutes at 400g and the plasma was collected and stored at -70 °C. The remaining sample was diluted 1:1 in RPMI and carefully layered onto a 4 ml of Nycoprep density gradient and centrifuged for 30 minutes at 400g at room temperature, centrifuge brake turned off. PBMC were transferred into a fresh tube, washed twice in RPMI 1640 (10 minutes, 400g), and resuspended at a concentration of 1×10^6 cells/ml in complete culture medium (RPMI 1640, 10% human serum (Sigma), 100U/ml penicillin (Sigma), 100 μ g/ml streptomycin (Sigma), and 2mM L-glutamine (GIBCO/Invitrogen, Paisley, UK).

The cells were rested for two hours at 37°C after which they were used for overnight stimulations and measurement of intracellular cytokine staining and activated T cells as described below.

3.7 Intracellular cytokine staining for production of HIV-specific CD4 and CD8 T cells

The levels of HIV-1 and HIV-2 specific CD4 and CD8 T cells were evaluated using overnight stimulation followed by intracellular cytokine staining. Overlapping Gag HIV-1 peptides based on consensus sequences of Clade A (15 mers overlapping by 10) were obtained from the National Institute of Health AIDS Research and Reference Reagent program. Peptides were dissolved in DMSO (Sigma-Aldrich) at 100mg/ml and diluted with RPMI to a final concentration of 20mg/ml before being individually filtered. The peptides were pooled, and aliquots stored at -70°C. HIV-2 Gag peptide pools (18 mers overlapping by 10) based on the consensus sequence from Clade A were kindly donated by Dr Aleksandra Leligdowicz (Leligdowicz *et al* 2007).

1×10^6 cells were stimulated with CD28/CD49d alone (negative control), Gag peptide pools (2µg/ml) or SEB (2µg/ml, positive control). The CD107a-PE antibody (a marker of cellular degranulation) was added at time 0. After 3 hours, 10µg/ml of protein transport inhibitor, brefeldin A (BD Biosciences, USA), was added and cells were incubated overnight. At the end of the incubation, cells were washed and permeabilised for 20 minutes at 4°C with the Fix/ Perm Buffer diluted in 1:4 with diluents provided by the manufacturer (Ebiosciences, US). Cells were then washed and incubated with 2% rat serum for 15 mins to reduce non-specific binding of antibodies. Cells were then simultaneously stained with six antibodies using the following panels: IFN-γ- FITC, CD4 PerCP, CD8 PE-Cy7, IL-2 APC and FOXP3-PB. All conjugated antibodies were obtained from Ebiosciences, while the PerCP conjugated antibodies were purchased from BD Biosciences. Cells were acquired

on the Cyan flow cytometer which was fitted with three lasers (488nm (Blue), 405nm (Violet) and 635nm (Red)). Unlike the FACSCalibur which is only fitted with two laser and can measure only four parameters, the Cyan machine can simultaneously measure emissions from up to nine fluorochromes. A minimum of 300,000 events were collected for each sample.

3.8 Measurement of T cell activation marker expression

Expression of the CD38 (cyclic ADP ribose hydrolase) on T cells was shown to be an accurate marker on activation which correlated with progression to AIDS (Giorgi *et al.*, 1993 & 1999). In addition, HLA-DR, an MHC Class II molecule measured in conjunction with CD38 was shown to be raised on CD8 T cells in HIV-1 infections compared to healthy controls (Kestens *et. al*, 1992). To determine the levels of immune activation in the T cell compartment in HIV-1 and HIV-2 patients freshly isolated PBMC (1×10^6 cells) were used for determination of T cell surface activation marker expression using anti-HLA-DR PerCP (BD Biosciences, USA), CD38 APC, CD4 PerCP, CD8 FITC, CD127 PE and FOXP3 PB titrated monoclonal antibodies (Ebiosciences, USA). Samples were analysed using a 9-Colour Cyan (Becton Dickinson, USA) and data was analyzed using FlowJo (Tree Star).

3.9 Depletion of Tregs from PBMCs using positive selection of CD25+ cells

CD25+ cells were selectively removed using magnetic bead cell sorting (MACS) from Miltenyi. Freshly isolated PBMC (minimum of 6 million cells) were split into two equal aliquots. One aliquot was labeled as the undepleted fraction and rested at 37°C until required. PBMC from the second aliquot were washed and resuspended in FACS Buffer (Phosphate Buffered Saline (Sigma, UK) containing 2% BSA and 0.5mM EDTA). CD25+ beads were added at a ratio of 5:1 to cells and incubated for 20 mins at 4°C. Cells were then pelleted (3000g, 5 mins) and resuspended in 2 ml of FACS Buffer. The cells were then put through a MACS column attached to a magnet. The column was washed three

times with FACS buffer and the flow-through was collected as the CD25⁻ fraction. CD25⁺ cells were obtained by adding 1 ml of FACS Buffer and flushing the column using the plunger provided. The cells in the CD25⁻ and CD25⁺ fraction were resuspended in complete media and counted before they were rested at 37°C. An aliquot of the CD25⁺ and CD25⁻ fractions were stained with CD25 FITC, CD127 PE, CD4 PerCP and FOXP3 to determine purity of the samples.

3.10 RNA Extraction

RNA was extracted from 2.5 ml of whole blood preserved in PAXGene tubes (Qiagen, UK) using the PAX gene Blood RNA Kit (Qiagen/BD, UK) as recommended by the manufacturer. The PAXGene tubes containing the whole blood samples were incubated at room temperature overnight to ensure lysis of cells and subsequently stored at -70°C. To extract RNA, PAXGene tubes were thawed and nucleic acids were pelleted by centrifugation (3000g, 10mins) and resuspended in buffers supplied by the manufacturer together with Proteinase K. The cell lysate was transferred to PAXgene Shredder spin column, centrifuged and supernatant collected in a fresh microcentrifuge tube. RNA was purified by passing it on to the PAXgene RNA spin column, then washed and treated with DNase. RNA was eluted in a final volume of 80µl and stored at -70°C. 1µl of each sample was used to quantify RNA levels using the Nanodrop spectrophotometer (Thermo Scientific, US). All RNA samples were stored at -70°C.

3.11 Real Time PCR

Real time PCR is a technique that allows for the simultaneous amplification and quantification of a region of DNA. Coupled with reverse transcription which converts RNA to DNA, this method provides a useful method to measure the relative expression of a particular gene. The expression of the gene of interest has to be normalized against the expression of constitutively expressed genes (housekeeping genes).

To measure FOXP3 expression in HIV-1 and HIV-2 infected patients an aliquot of blood was stored in a collection tube containing RNA stabilizing agents (PAXgene™ Blood RNA System, Pre-AnalytiX)) and stored at -70°C. RNA was isolated as described above. cDNA was synthesized using One step RT Reagents (Qiagen, UK) following the manufacturer’s instructions. The FOXP3 and Beta-glucuronidase (GUSB) assays were performed using the QuantiTect™ SYBR® Green PCR kit while the HUPO Probe assay was performed using the QuantiTect™ Probe PCR kit (Qiagen). Primers used for each assay are listed in Table 3.1.

Primer	Sequence	Annealing (°C)	Number of Cycles
FoxP3 F	5' ACCTGGAAGAACGCCATC 3'	58	40
FoxP3 R	5' TGTCGTCCATCCTCCTTTC 3'		
HuPO-F	5' GCTTCCTGGAGGGTGTCC 3'	56	40
HuPO-R	5' GGACTCGTTTGTACCCGTTG 3'		
HuPO probe	5'FAM-TGCCAGTGTCTGTCTGCAGATTGG-TAMRA 3'		
GUSB-F	5' AAA CGA TTG CAG GGT TTC AC 3'	55	40
GUSB-R	5' CTC TCG TCG GTG ACT GTT CA 3'		

Table 3.1: Primers pairs and conditions for real time PCR assays

For each individual assay a standard curve (range 10⁶- 10¹ copies) was generated using gel purified PCR products. Each individual sample and standards were run in duplicate and the results were displayed as mean copy numbers. All assays were performed on the DNA Engine Opticon® Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA), and analyzed using Opticon Monitor 2™ analysis software.

3.12 Quantitation of cytokines in Human Plasma

Multiplex quantification of cytokine concentrations in plasma samples was performed using the Bio-plex® 200 system (Bio-Rad Laboratories). This commercially available assay allows for the simultaneous measurement of cytokine levels in plasma samples, which rely on antibody conjugated beads. Levels of IL-2, IL-6, IL-10, IL-17, IL-12, IFN- γ , MIP-1 β and TNF- α were determined using the x-Plex™ assays according to the manufacturer's instructions (Bio-Rad Laboratories). Data analysis was performed using the Bio-Plex® Manager software (Bio-Rad Laboratories). The lower limit of cytokine detection was defined as the concentration corresponding to a fluorescence value 3SD above the mean background fluorescence in control wells (IL-2 3.99 pg/ml, IL-10 3.56 pg/ml, IL-17 43.22 pg/ml, IL-12 3.99 pg/ml, IFN- γ 7.58 pg/ml and TNF- α 8.24 pg/ml); values above this threshold were designated as responders, values below were arbitrarily set to the threshold value.

3.13 Statistics

Data was analyzed using Stata software Version 9.2. Kruskal-Wallis test was used to analyze the medians of non-normally distributed variables between groups. For linear regression and calculation of Pearson's correlation coefficients variables were log transformed to satisfy the condition of normal distribution when using these statistical tests. The non-parametric Spearman's' test was used to assess correlation on non-normally distributed data. Statistical significance was defined as $p < 0.05$.

Chapter 4: Phenotype and quantification of T regulatory cells in HIV infected patients and healthy controls

4.1 Introduction

The immune system has multiple checks to ensure a controlled response in order to avoid host damage. The idea that the immune system possesses an endogenous subset of T cells that are professional suppressors was suggested in the 1970s (Gershon and Kondo, 1970) but interest in this field waned due to a failure to identify a cellular marker that defined these cells. The finding that CD4⁺ T cells expressing the α -chain of the IL-2 receptor (CD25) possessed suppressor activity re-ignited interest in the field and led to the rebirth of interest in suppressor T cells renamed as T regulatory cells (Tregs), (Sakaguchi *et al.*, 1995). This was given more impetus by the finding that expression of the transcription factor forkhead box protein P3 (FOXP3) was essential for suppression, thus providing an additional and more precise marker to define these cells (Fontenot *et al.*, 2003, Hori *et al.*, 2003, Khattri *et al.*, 2003). However since FOXP3 is an intracellular marker which required fixing and permeabilising cells, it was necessary to identify other cellular markers to allow functional studies of these cells. It was observed that the IL-7 receptor was down regulated on CD4⁺FOXP3⁺ cells and showed an inverse correlation with expression levels of FOXP3 (Liu *et al.*, 2006; Seddiki *et al.*, 2006) and this provided an additional useful marker.

The caveat for the use of markers attributed to Tregs (CD25, HLA-DR, low CD127) has been that they are also present on activated T cells. During HIV infection, the immune system is chronically activated which results in an increase in activation markers such as CD25, HLA-DR and down regulation of CD127, all of which can be expressed on CD4⁺FOXP3⁺ cells. Therefore identifying the optimal signature to define Tregs in HIV studies has been a challenge.

More recent studies have indicated that CD4⁺FOXP3⁺ cells represent a heterogeneous population of naive and memory Tregs that are equally suppressive but display different homing and functional markers such as CTLA-4(Cytotoxic T-Lymphocyte Antigen-4, CD152) and CD39(Ectonucleosidetriphosphate diphosphohydrolase 1)(Deaglio *et al.*, 2007, Miyara *et al.*, 2009). CD39 hydrolyzes ATP/UDP and ADP/UDP to generate nucleoside monophosphates, which can be degraded further to nucleosides such as adenosine. The generation of adenosine by CD39 enzymatic activity has been demonstrated to suppress proliferative responses (Ohta *et al.*, 2001). CTLA-4 is another negative regulator of T cell function and competes with CD28 for ligation with CD80 and CD86 on antigen presenting cells. CTLA-4 ligation results in decreased IL-2 production, inhibition of cell cycle progression and modulation of TCR signaling (reviewed by Bluestone *et al.*, 2008).

Memory Tregs (defined by the expression of CD45RO) are the predominant subset of Tregs; and express CTLA-4, CD25, GITR and CD95 in adults (Sakaguchi *et al.*, 2004). These cells preferentially home to tissue as they express high levels of the chemokine receptor CCR4 and are very prone to apoptosis induced cell death (Fritzsching *et al.*, 2005a). In contrast naive Tregs (CD45RA⁺ FOXP3⁺) cells express CCR7 and home to the lymph nodes and express low levels of CD25 and CTLA-4 as well as FOXP3 (Fritzsching *et al.*, 2005b). The majority of these cells also express CD31 (Platelet endothelial cell adhesion molecule (PECAM-1)) which indicates that they are recent thymic emigrants (Booth *et al.*, 2010). Interestingly, naive Tregs are relatively resistant to CD95L mediated apoptosis compared to memory Tregs, but upon stimulation can rapidly convert to memory Tregs (Fritzsching *et al.*, 2006).

Faced with such diversity in classification, it is therefore not surprising that the role of Tregs in HIV remains a contentious subject. Many of the early studies exploring Tregs in HIV infection were marred by inconsistencies relating to the phenotype used to enumerate these cells. More specifically, the use of CD4⁺CD25⁺ before conjugated antibodies to FOXP3 became available, has resulted in erroneously defining activated cells as Tregs. Other studies utilized CD4⁺CD25^{hi} to define Tregs (Kinter *et al.*, 2004), and while this population has been shown to contain more FOXP3⁺ cells, it remains an imprecise definition. More recent studies have identified Tregs as CD4⁺FOXP3⁺ CD4⁺FOXP3⁺CD25⁺, CD4⁺CD127^{lo/-}FOXP3⁺ or CD4⁺CD25⁺CD127^{lo} (Krathwohl *et al* 2006; Eppl *et al.* 2006; Kinter *et al.* 2007; Lim *et al.*, 2007; Seddiki *et al.*; 2009,).

Before the availability of conjugated monoclonal antibodies against FOXP3, many studies using HIV subjects measured the messenger RNA (transcript) levels of *FOXP3* as a surrogate marker for frequency of Tregs (Oswald-Richter *et al.*, 2004; Kinter *et al.*, 2004; Weiss *et al.*, Eggena *et al.*, 2005; Apoil *et al.*, 2005). However, as yet there remain no reports that have evaluated whether FOXP3 transcript levels correlate with frequencies of CD4⁺FOXP3⁺ cells measured using flow cytometry.

In this chapter the optimal combination of markers to measure Tregs using flow cytometry was explored. The proportion of naïve and memory Tregs of the total CD4⁺FOXP3⁺ in HIV-1, HIV-2 and healthy controls was also determined. The levels of CD4⁺FOXP3⁺ cells, as measured by flow cytometry, was also compared to quantitative real-time PCR measurement of *FOXP3* transcript levels.

4.2 Materials and methods

4.2.1 Subjects

Whole blood was collected from HIV-1, HIV-2 infected subjects and healthy controls individuals attending the GUM Clinic at the MRC Laboratories in The Gambia as described in Chapter 3 (Materials and methods).

4.2.2 Phenotyping and Quantification of Tregs using flow cytometry

To determine the appropriate surface and intracellular markers to identify Tregs, aliquots of whole blood were stained with the following panels of antibodies:

- 1) CD25-FITC, CD127-PE, CD4-PerCP, FOXP3-APC
- 2) CD39-FITC, CD27-PE, CD4-PE-Cy7, CD45-RO PE-Cy5, CD28-APC, FOXP3-PB
- 3) CD39-FITC, CTLA-4 PE, CD4 PE-Cy7, HLA-DR PerCP, CD25-APC, FOXP3-PB

With each run an isotype control for FITC was included in order to define the cut-off for CD39 and CD25 positive staining. All samples were acquired on a 9-color Cyan machine (Beckman Coulter, USA) and a minimum of 100,000 events were collected in the CD4 gate. The FCS files were analyzed using FlowJo software version 7.2.5

4.2.3 Quantitative Real-time PCR

RNA was extracted from whole blood collected from study samples in PAXGene tubes (Qiagen, UK) according to the manufacturer's recommendations. An aliquot of RNA was reverse transcribed to copy DNA (cDNA) and the levels of FOXP3 mRNA were measured as described in the Materials and methods chapter (Chapter 3).

4.2.3 Statistics

Data were analyzed using Stata software Version 11 (Stata Corp, USA) and data graphs were prepared using Prism (v5). Correlations were tested using Spearman's rank correlation co-efficient for non-normally distributed data. Statistical test results were considered significant if $p < 0.05$.

4.3 Results

4.3.1 Definition of Tregs in HIV infections using flow cytometry:

The combination of cell surface and intracellular markers used to quantitate Tregs in HIV studies has varied between studies. FOXP3 is required for the maintenance and function of Tregs (Fontenot *et al.*, 2003). Thus we sought to optimize Treg identification by using a combination of appropriate cell surface markers in conjunction with FOXP3 in direct whole blood staining, rather than using more purified PBMC. Such a method required less blood (see fig 4.1).

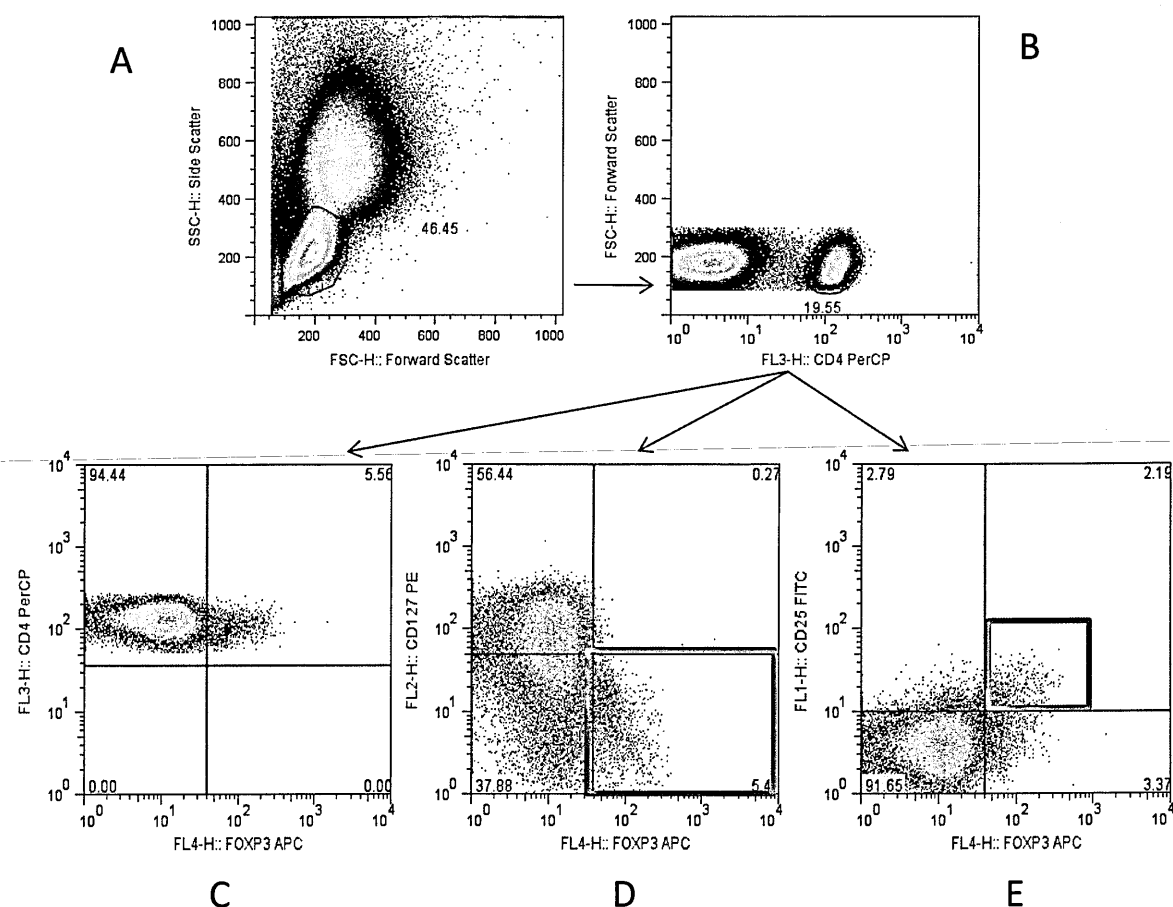


Figure 4.1: Gating strategy to enumerate Tregs in whole blood samples: A) Lymphocyte gate (Forward scatter versus Side scatter) B) CD4 gate (CD4 versus Forward Scatter) C) CD4 versus FOXP3 within the CD4 gate D) CD127 versus FOXP3 within the CD4 gate E) CD25 versus FOXP3 within the CD4 gate

A combination of antibodies that specifically bind CD4, CD127 (α chain of the IL-7 receptor), CD25 (α chain of the IL-2 receptor), and FOXP3 was used to define Tregs. All CD4⁺ cells that expressed FOXP3 displayed low levels of CD127 expression and the expression levels of these two markers was inversely correlated (Figure 4.1D). All CD4⁺FOXP3⁺ cells showed low levels of CD127 expression in healthy controls and in HIV infected individuals.

Having shown that CD127 and FOXP3 expression identifies a discernable population of CD4 cells, the expression of CD25 was examined in this population. The cut-off to define CD4 T cells positive for CD25 was determined using an isotype control. (Figure 4.2)

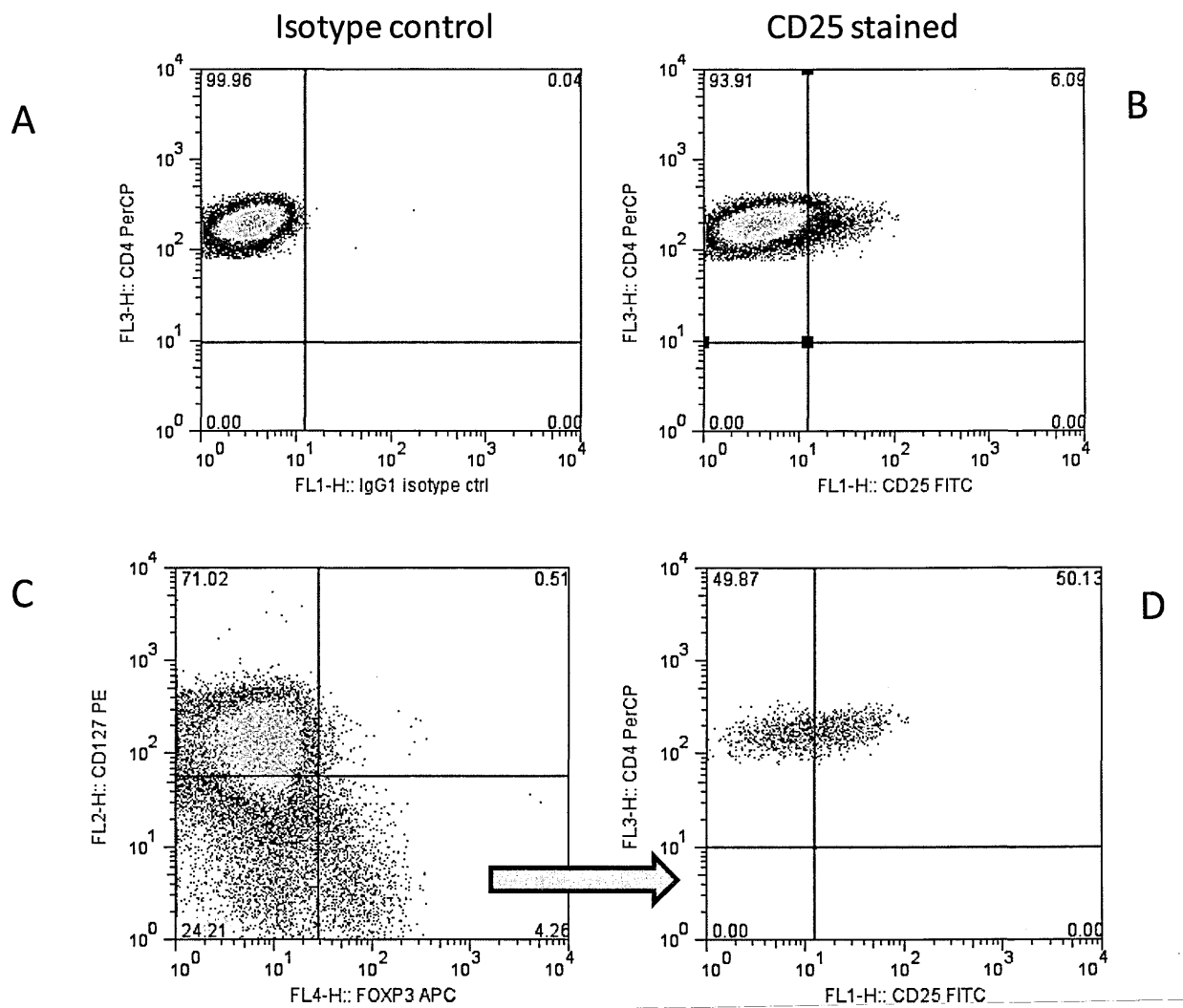


Figure 4.2: $CD4^+FOXP3^+CD127^{lo/-}$ encompass $CD25^+$ and $CD25^-$ Tregs: A) Determination of $CD25^+$ cut-off within the CD4 gate using a isotype control for FITC B) Cross hairs from A copied onto CD25 FITC stained sample C) CD127 versus FOXP3 D) CD25 expression on $FOXP3^+$ events from C.

Using this gating strategy, it was evident that CD4⁺FOXP3⁺CD127^{lo/-} cells included both CD25⁺ and CD25⁻ cells in healthy individuals and HIV infected patients as well (Fig 4.2B). In addition it was clear that the levels of CD4⁺FOXP3⁺CD127^{lo/-} showed a strong positive correlation with CD4⁺FOXP3⁺CD25⁺ ($r = 0.7123$, $p = 0.0001$).

4.3.2 State of differentiation of Tregs

The enumeration of CD4⁺ FOXP3⁺ cells was performed in samples from healthy controls and in HIV-1 and HIV-2 subjects to determine the proportion of Tregs that displayed surface markers consistent with a naive or memory phenotype. Using CD27 and CD45RO markers, naive (CD27⁺CD45RO⁻), early differentiated (CD27⁺CD45RO⁺) and late differentiated (CD27⁻CD45RO⁺) Tregs were detected within the CD4⁺FOXP3⁺ population. The majority of CD4⁺FOXP3⁺ cells were found to display an early differentiation phenotype in both HIV infected subjects and controls. However a sizeable proportion of these cells were also found within the CD27⁺CD45RO⁻ population, which is consistent with a naive phenotype (Figure 4.3A). These naive Tregs had markedly lower levels of CD28 and FOXP3 expression than CD27⁺CD45RO⁺ Tregs. This was evident in both HIV infected and healthy control samples (Figure 4.3B). Having shown that CD4⁺FOXP3⁺ cells represent a heterogeneous population of Tregs with both high and low expression of CD28/FOXP3, we next determined whether the level of expression could be linked to a functional difference by staining with CD39. Activity of CD39 in Tregs has been shown to result in suppression of proliferative responses from effector cells. The expression of CD39 was significantly higher on CD4⁺ FOXP3⁺ CD45RO⁺ compared to CD4⁺ FOXP3⁺ CD45RO⁻ cells (Figure 4.3D).

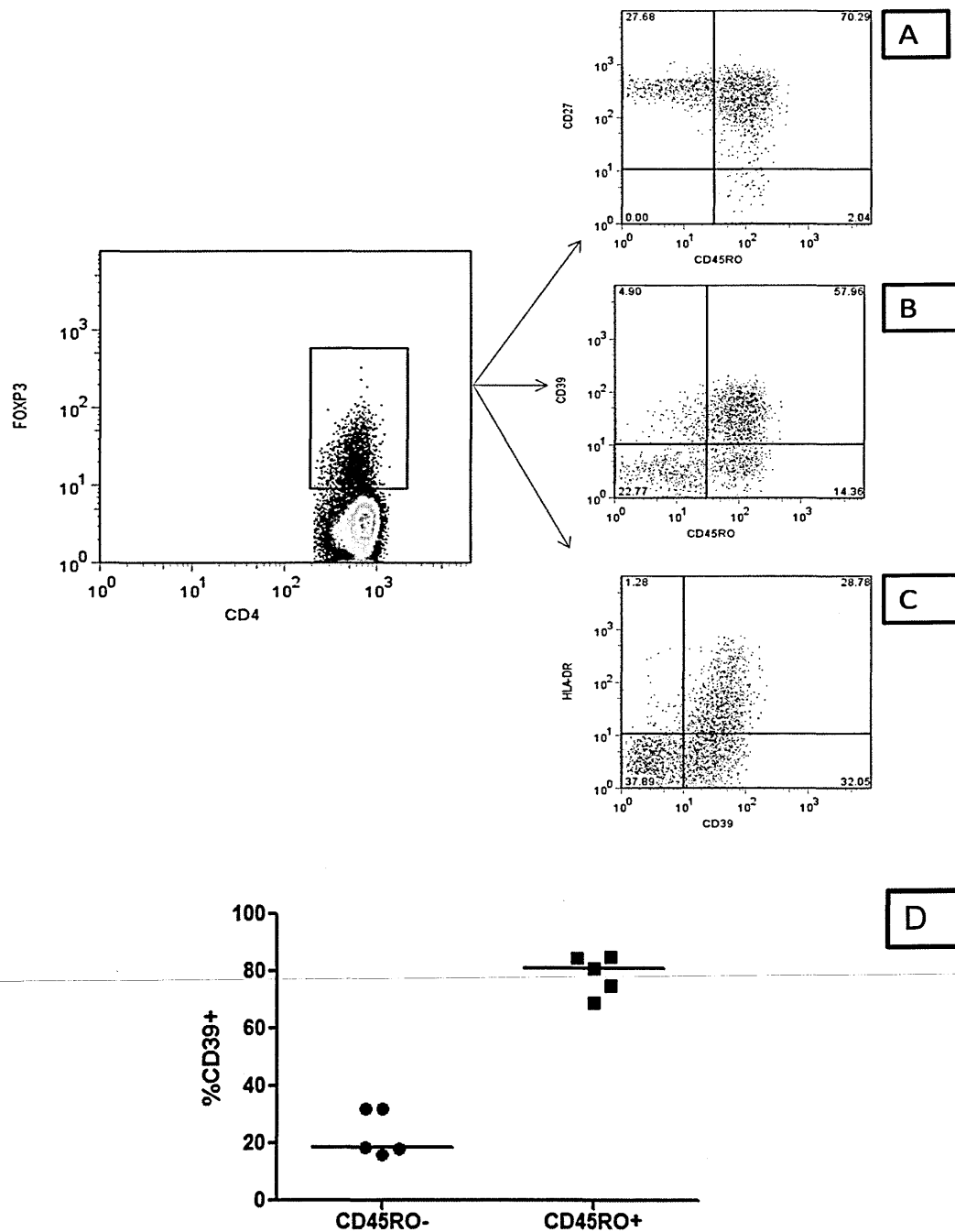


Figure 4.3: CD4⁺FOXP3⁺ represents a heterogeneous population of T cells with a naive and memory phenotype. A) Proportion of CD4⁺FOXP3⁺ cells are a mix of naive, early differentiated and late differentiated cells B) CD39 expression is restricted to CD45RO⁺ cells within the CD4⁺FOXP3⁺ cells C) HLA-DR⁺ cells within the CD4⁺FOXP3⁺ gate express CD39 D) Comparison of CD39 expression on CD45RO⁺ and CD45RO⁻ Tregs in healthy controls.

The proportion of naive Tregs ($CD4^+ CD45RO^- CD27^+ FOXP3^+$) were compared between HIV-1, HIV-2 and healthy controls. There was no significant difference in the proportion of $CD4^+ FOXP3^+$ cells that displayed a naive phenotype between HIV-1 (median = 25.3(IQR = 15.5-34.2)), HIV-2 (median =23.2 (IQR = 13.8-37.2)) infected subjects and healthy controls (median = 17.75 (IQR = 8.8-17.8)), $p=0.12$, Figure 4.4B).

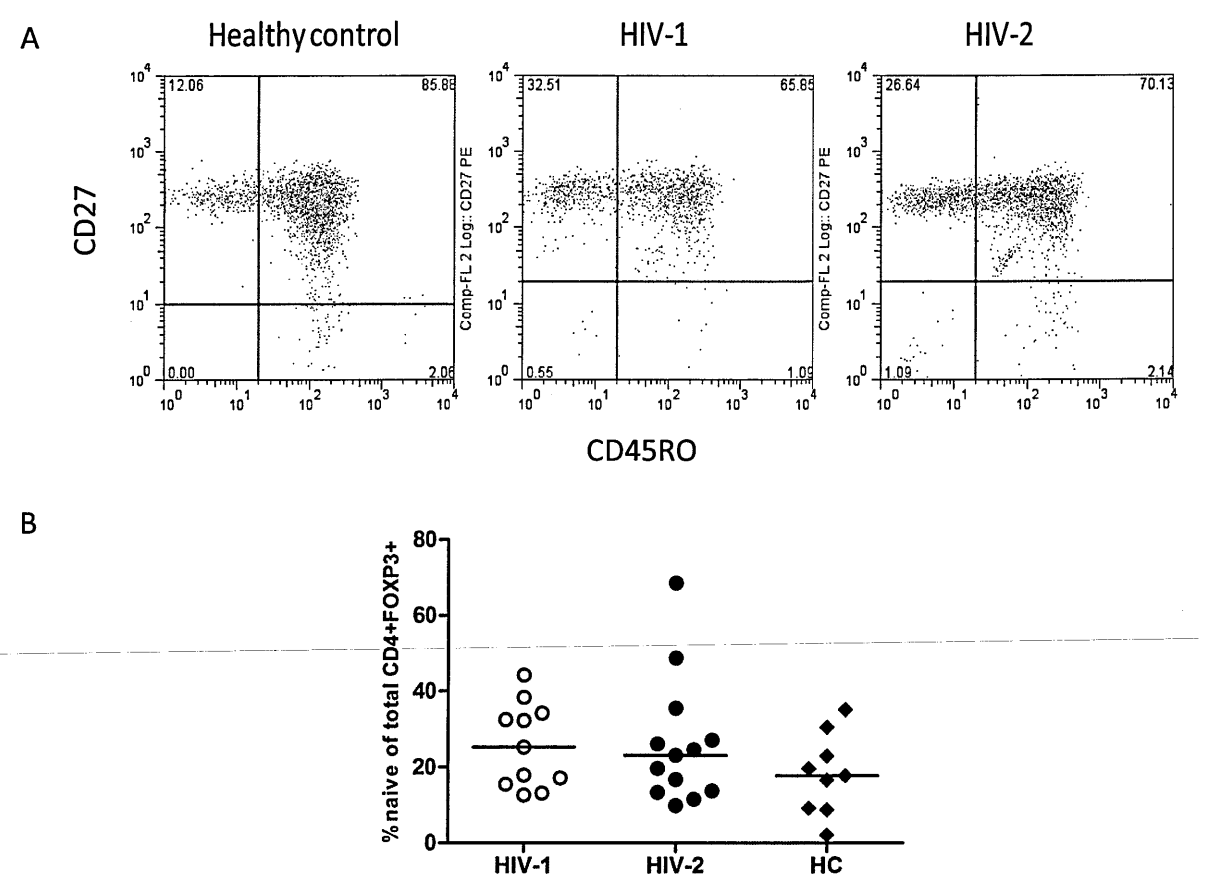


Figure 4.4: Comparison of the proportion of $CD4^+ FOXP3^+$ T cells that display a naive phenotype ($CD45RO^- CD27^+$) in HIV-1, HIV-2 and healthy controls. A) Representative plots of the distribution of $CD4^+ FOXP3^+$ in the naïve ($CD27^+ CD45RO^-$), early differentiated ($CD27^+ CD45RO^+$), Late differentiated($CD27^- CD45RO^+$), and terminally differentiated ($CD27^- CD45RO^-$) subsets in HIV-1, HIV-2 and healthy controls. B) Comparison of the percentage of $CD4^+ FOXP3^+$ cells with a naïve phenotype in HIV-1, HIV-2 and healthy controls. Data was analyzed using the non-parametric Mann-Whitney U test.

4.3.4 Comparison of *FOXP3* transcript levels and quantification by flow cytometry of *CD4⁺FOXP3⁺* cells:

Real-time PCR measures the total mRNA content of a sample while flow cytometry allows the identification of cells expressing particular cell surface and intracellular proteins. As yet it is unclear whether the output from these two different methodologies is comparable when measuring *FOXP3*.

In this section *FOXP3* transcript (mRNA) levels were measured using reverse transcription coupled with real-time PCR and then compared to the number of *CD4⁺FOXP3⁺* cells measured using flow cytometry. *FOXP3* transcript levels were normalized against the transcript levels of the housekeeping gene *beta-D-glucuronidase (GUSB)*.

Interestingly, we did not find any significant correlation between the percentages of *FOXP3* positive cells measured by flow cytometry and transcript levels of *FOXP3* mRNA in our study samples (Figure 4.5A and 4.5B). To determine if aberrant normalization of RT-PCR values could account for the discrepancy between RT-PCR and FACS results, the RT-PCR data were normalized to a second housekeeping gene, *RPLP0*. Again no correlation between the percent or absolute frequency of *CD4⁺FOXP3⁺* and transcript levels of *FOXP3* was observed (Figure 4.5C and 4.5D).

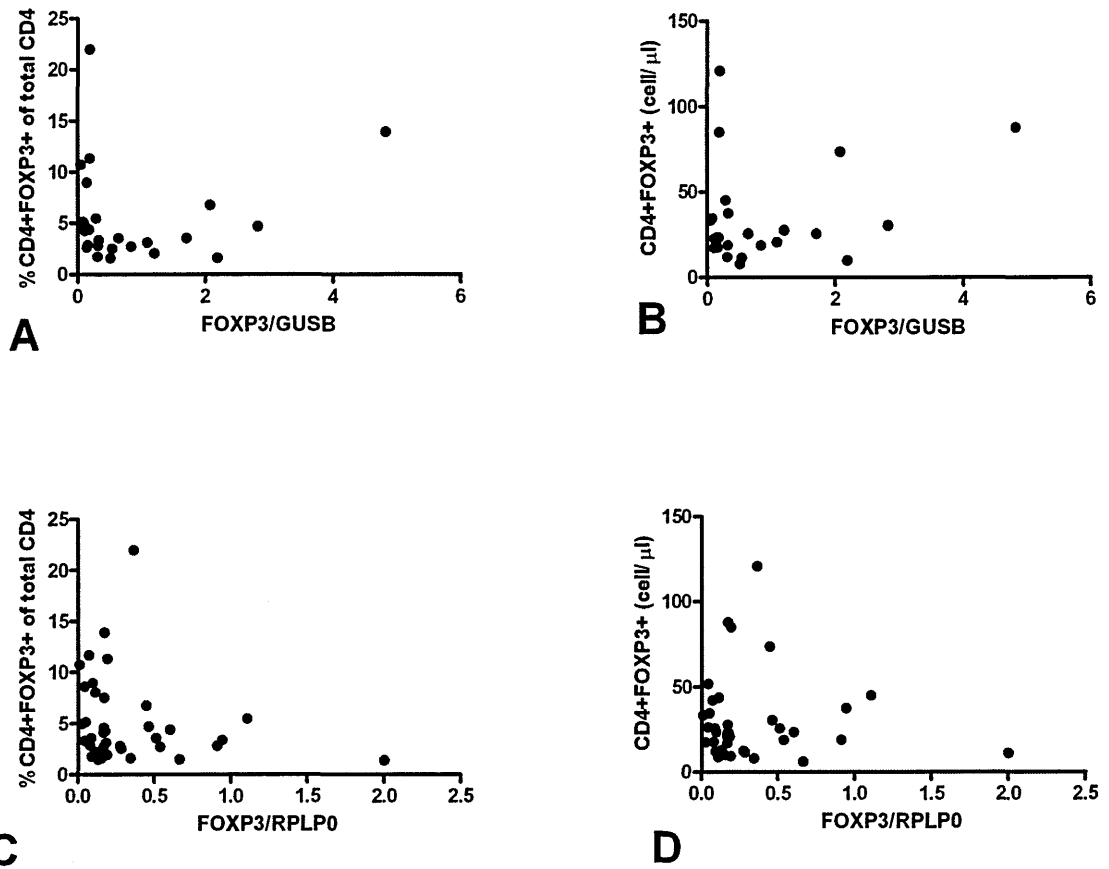


Figure 4.5: Comparison between frequency of CD4⁺FOXP3⁺ and transcript levels of FOXP3 in HIV Patients : A) Percent Treg levels versus *FOXP3* transcript levels normalized using *GUSB* B) Absolute Tregs levels versus *FOXP3* transcript levels normalized using *GUSB* C) Percent Treg levels versus *FOXP3* transcript levels normalized using *RPLP0* D) Absolute Tregs levels versus *FOXP3* transcript levels normalized using *RPLP0*. Correlations were assessed using the non-parametric Spearman's Rank correlation statistic

4.4 Discussion

Defining the Treg phenotype in HIV disease has remained a contentious subject which maybe a contributing factor in the variable conclusions about the role of Tregs in helping or hindering the immune system during HIV infection. The observation that FOXP3 expression in CD4 T cells correlated with the ability of these cells to suppress key immune functions in the infected host was a major step forward. (Liu *et al.*, 2006; Seddiki *et al.*, 2006; also see figure 1C). Similarly, the observation that CD127 expression inversely correlated with FOXP3 expression provided an additional marker to precisely identify these cells (also see figure 1D). Using a four-color flow cytometry staining panel, it was clear that the use of CD127 and FOXP3 defined a clear population of CD4 T cells that can be objectively enumerated (figure 4.1D). The gating strategy that used CD4⁺CD25⁺ alone to measure Tregs has been shown to be inaccurate since a large proportion of CD4⁺FOXP3⁺ do not express CD25. Indeed, it has been demonstrated here that the CD4⁺CD127^{lo/-}FOXP3⁺ population of cells is heterogeneous with regards to the level of CD25 expression (Figure 4.2D). This was evident in healthy controls as well as HIV infected patients. Therefore enumeration of Tregs using CD4⁺CD127^{lo/-}FOXP3⁺ represents a robust gating strategy to identify the total Treg population.

As interest in FOXP3 expressing Tregs has increased it has become important to develop better phenotypic and functional markers to define these cells. The analysis in this chapter revealed that the majority of Tregs in HIV-1, HIV-2 and healthy controls displayed a cellular phenotype consistent with early differentiated cells characterized by the simultaneous surface expression of CD45RO, CD27 and high levels of CD28. However, within these CD4⁺FOXP3⁺ cells there was a population of CD45RO⁻, CD27⁺ and CD28^{lo} cells consistent with a naive phenotype. The expression of CD39 was significantly higher on CD45RO⁺ Tregs compared to CD45RO⁻ Tregs in HIV infected and in healthy individuals

which is consistent with previous suggestions that these cells represent activated Tregs. Indeed recent reports have shown the CD45RO⁺ Tregs were highly proliferative when compared to CD45RA⁺ Tregs and homed to different tissues and expressed significantly higher levels of CD39 (Miyara *et al.*, 2009; Booth *et al.*, 2010). Importantly, the current study also found that CD39 expression was present on all HLA-DR⁺ FOXP3⁺ cells. Studies have shown that HLA-DR expressing Tregs were more potent suppressors of effector immune responses (Baecher-Allan *et al.*, 2009). Thus, although we did not assess HLA-DR and CD45RO expression in the same panel, the co-expression of HLA-DR on CD39⁺ FOXP3⁺ cells suggests that they possess a memory phenotype.

The proportion of Tregs with a naive phenotype was similar in HIV infected individuals compared to healthy controls, and no differences were seen when comparing HIV-1 and HIV-2 subjects. It has been suggested that naive Tregs are highly resistant to apoptosis and upon stimulation can quickly convert to a memory phenotype (Booth *et al.*, 2010).

This would be an advantageous strategy in a setting of chronic stimulation and maybe required to counter the high levels of systemic immune activation observed during HIV infections.

A comparison of flow cytometry analysis of CD4⁺FOXP3⁺ cells and transcript levels of *FOXP3* using quantitative real-time PCR was also explored. The results of the two methods did not correlate which strongly suggest that not all FOXP3 transcripts are translated to proteins. This is consistent with a previous study that showed that although *FOXP3* transcript levels were readily detected in several non-T cell lines, protein expression was not present (Yamamoto *et al.*, 2008).

In summary, the results of this chapter show that CD4⁺FOXP3⁺CD127^{lo/-} can be used to measure the total Treg population, which encompasses both CD25⁺ and CD25⁻ Tregs.

Furthermore, Tregs exhibit a predominantly memory phenotype (CD27⁺CD45RO⁺) but a sizeable minority of these cells display a naive phenotype (CD27⁺CD45RO⁻) which maybe in a resting state. Finally it was demonstrated that enumeration of Tregs by flow cytometry provides values that are different from those of *FOXP3* mRNA levels. Therefore, caution is warranted in interpreting the results of studies which used *FOXP3* transcript levels in whole blood as a surrogate for total Treg numbers.

Chapter 5: Comparison of T regulatory cells in HIV-1 and HIV-2 infection and relationship to viremia and systemic immune activation

5.1 Introduction

Both HIV-1 and HIV-2 infections are found in West Africa but their natural history in man differs markedly. The mortality risk in HIV-2 infected individuals is shown to be a mere two fold higher when compared to uninfected controls, while HIV-1 infected individuals have a 10-20 fold increase in mortality (Jaffar *et al.*, 2004). The two viruses share significant homology in their genomes and primarily infect CD4 T cells (Guyader *et al.*, 1987). However the majority of HIV-2 subjects maintain high CD4 T cell counts and low or undetectable peripheral viremia (Ariyoshi *et al.*, 2000; Berry *et al.*, 1998, Jaffar *et al.*, 1997). The reason for the marked difference in plasma viremia between HIV-1 and HIV-2 (Popper *et al.*, 1999, Berry *et al.* 2002) despite a similar pro-viral load (Ariyoshi *et al.*, 1996; Popper *et al.*, 2000) in these subjects is not clear. It may be the result of an enhanced immune control of viral replication, defective HIV-2 replicative capacity, or a combination of both these mechanisms.

The clinical course of HIV-2 infection has been considered as a unique model of an attenuated HIV-1 infection. However, some HIV-2 infected subjects with high viral load succumb quickly in a manner indistinguishable from HIV-1 (van der Ende *et al.* 1996), suggesting that the virus may not necessarily be less pathogenic by itself. Thus, understanding the immunopathogenic basis of the differences between HIV-1 and HIV-2 and between HIV-2 progressors and non progressors may be very relevant for the design of an HIV-1 vaccine.

Evidence has begun to emerge that HIV-2 asymptomatic patients exert more efficient immune responses than their HIV-1 counterparts as shown by the presence of strong polyfunctional T cell responses expressing the cytotoxic molecules granzymes and perforin as well as the cytokines interleukin-2 and interferon- γ (Duvall *et al.*, 2006 2008). Innate immune responses notably NK cell activity are greater in HIV-2 subjects with high

CD4 counts compared with HIV-1 patients with comparable CD4 levels (Nuvor *et al.*, 2006). Other studies have recently demonstrated that strong HIV-2 specific cellular immune responses inversely correlated with plasma viremia (Leligdowicz *et al.*, 2007). Together these studies suggest that the immune response is maintained in HIV-2 subjects and may account for long term non progression in majority of infection. However, the precise reason for their demise in some patients remains unclear. Added to this efficient immune response may be an effective immune regulatory mechanism, which hitherto is unexplored in HIV-2. The evidence of the role of a suppressive subset of CD4 T cells, termed T regulatory cells (Tregs) in viral infection (Reviewed in Rouse *et al.*, 2006) raised the possibility that the immune response in HIV-2 infections was more efficiently regulated thus preventing immunopathology.

The role of Tregs in HIV pathogenesis has yet to be resolved. Studies have shown that Tregs restrain the HIV specific cellular responses needed to control the virus both in the periphery (Kinter *et al.*, 2007) and in lymphoid tissues (Kinter *et al.*, 2007b, Epplé *et al.*, 2006, Nilsson *et al.*, 2006). Other authors suggest that regulatory function is associated with favourable clinical outcome (Kinter *et al.*, 2004) and the presence of Tregs correlates with non- progression of disease because of their ability to curtail immune activation, a hallmark of HIV infection (Eggena *et al.*, 2005, Ndhlovu *et al.*, 2008, Card *et al.*, 2009). It is thus unclear whether Tregs play a protective role in HIV-1 infection by limiting the intense and harmful hyper-activation that is caused by high levels of replicating virus or whether their role is detrimental as they suppress T cell responses making them less efficient at controlling HIV replication.

A comprehensive analysis of Tregs in HIV-2 infection has yet to be conducted. This study compares Treg levels in ART naive HIV-1 and HIV-2 infected individuals in West Africa and correlates with viral load and immune activation in both patient groups.

5.2 Materials and Methods:

5.2.1 Study participants

After informed consent was obtained, 86 HIV-1 and 72 HIV-2 antiretroviral naive subjects were recruited from a clinic-based cohort at the Medical Research Council (MRC) Laboratories in The Gambia. HIV status of the patients was confirmed with HIV-1/ HIV-2 Murex ICE HIV-1-1.2.0 capture enzyme immunoassay (Murex Diagnostics, Kent, UK). Patients were grouped according to CD4⁺ T cell counts as high (≥ 500 cells/ μ l) or low (< 500 cells/ μ l). CD4 counts and viral load were measured as described in the Chapter 3. The Gambia Government/ MRC Ethics committee approved the study.

5.2.2 Phenotyping and Enumeration of Tregs

Tregs were measured on freshly taken whole blood using four colour flow cytometry as described in Chapter 3 (section 3.4).

5.2.3 Quantitation of soluble β -2 microglobulin:

Soluble immune activation biomarker β 2-microglobulin (β 2m) was determined by use of an automated microparticle EIA (AxSYM system, Abbott Laboratories) as described (Chapter 3.5).

5.2.3 Statistics

Data was analyzed using Stata software Version 11 (Stata Corp, USA) and Data graphs were prepared using Prism (version 5). The non-parametric Kruskal Wallis test was used to assess differences between groups. Correlations were tested using Spearmans rank correlation co-efficient for non-normally distributed data or Pearsons test for normally distributed data. Statistical test results were considered significant if $p < 0.05$.

5.3 Results

5.3.1 Patient characteristics:

Table 5.1 summarizes the demographic, CD4 and viral load data of HIV-1 and HIV-2 patients grouped by their absolute CD4 T cell counts.

The median age of all HIV-2 patients was 8.5 years greater than that of HIV-1 subjects. There was a high ratio of female: male in all groups, except in the HIV-1 patients with a low CD4 count. The viral loads were significantly lower ($p=0.0001$) in HIV-2 infected individuals compared with HIV-1 counterparts (median log viral load: 2.43 versus 4.17). HIV-2 subjects within the high CD4 group had a median log VL of 2.00, while that of the low CD4 groups it was 3.62 ($p=0.0001$).

Characteristic	CD4 ≥500 cells/μl		CD4< 500 cells/μl	
	HIV-1 (n=45)	HIV-2 (n=40)	HIV-1 (n=36)	HIV-2 (n=30)
Age in years	35 (30-53)	45	35 (30-43)	42 (30-50)
		(37-51)		
Female(%)	75	80	54	78
CD4%	27 (23-32)	35 (29-42)	19 (14.5-22)	23 (18-26)
Absolute CD4 count (cells/μl)	700 (580-880)	720 (620-870)	410 (350-440)	330 (270-400)
Log ₁₀ HIV-RNA (copies/ml)	3.98 (3.18-4.65)	2.00 (2-2.8)	4.49 (3.85- 5.26)	3.62 (2.44- 3.95)
Minimum duration of Infection(yrs)	2 (1-5)	8 (4-13)	1 (1- 3)	1.5 (1-9)

Table 5.1: Patient characteristics. Patients were grouped according to HIV status and absolute CD4 T cell counts. Median values are listed for each parameter with the interquartile ranges in brackets. The minimum duration of infection is the period between diagnosis and the time a patient was entered into this study.

5.3.2. Lower frequency of circulating Tregs levels in HIV-2 than HIV-1 infected subjects with a normal CD4+ T cell count.

HIV-2 patients with CD4 T cell counts of ≥ 500 cells/ μ l had significantly lower percentage of Tregs than HIV-1 patients (2.1 % (IQR: 1.0-3.4) versus 2.8 % (IQR: 1.9-4.7), $p=0.01$, Figure 5.1A). However, in those with a low CD4 count, there was no significant difference between the HIV-1 and HIV-2 patients (4.1% (IQR: 4.1-14.5) versus 3.6% (IQR: 1.8-6.1) $p=0.1181$). Percentage Treg levels in HIV-2 patients with high CD4 counts were also significantly lower than HIV-2 subjects with low CD4 T cell counts (median: 2.1 % (IQR: 1.0-3.4) versus 3.6% (IQR: 1.8-6.1), $p=0.03$). Similarly, HIV-1 patients with a high CD4 count had a lower percentage Tregs compared with those with a low CD4 count (median: 2.1 % (IQR: 1.0-3.4) versus 4.1% (IQR: 4.1-14.5), $p=0.02$)

A comparison of absolute numbers of Tregs in patients with a high CD4 count showed that HIV-2 infected patients had significantly lower numbers compared to HIV-1 (medians 16.7 cell/ μ l (IQR: 7.9-25.9)) versus 21.6 cells/ μ l (IQR: 13.1-35.5), $p=0.01$, Figure 5.1B). Similarly HIV-2 patients with low CD4 counts had lower numbers of absolute Treg compared to HIV-1 patients (medians: 9.1 (IQR: 5.4-20.4) versus 13.2 cells/ μ l (IQR: 10.4-23.7), $p=0.04$). Comparison of absolute Treg levels showed higher Treg levels in the high CD4 group compared to the low CD4 group in both HIV-1 (21.6 cells/ μ l (IQR: 13.1-35.5) versus 13.2 cells/ μ l (IQR:10.4-23.7) , $p=0.009$) and HIV-2 infection (medians 16.7 cell/ μ l (IQR: 7.9-25.9 versus medians: 9.1 cell/ μ l (IQR:5.4-20.4), $p=0.02$).

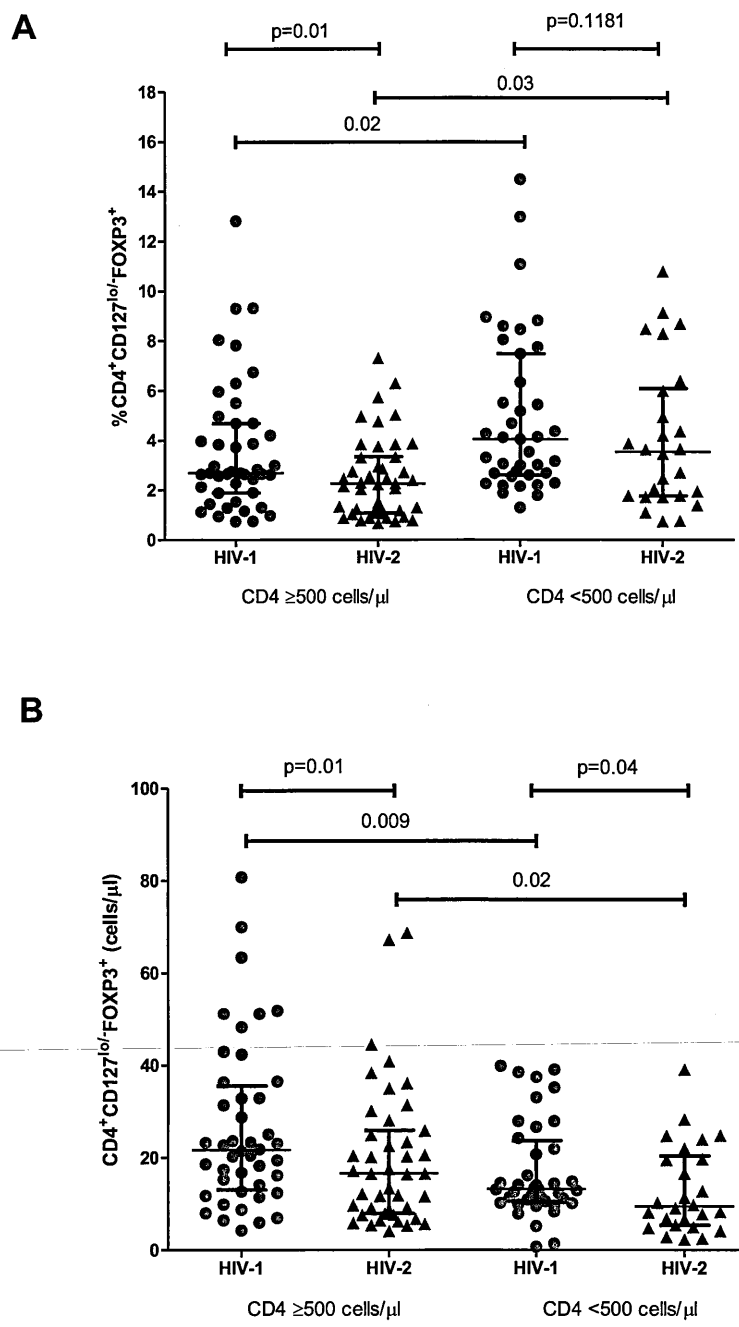


Figure 5.1: Percent and absolute levels of Tregs in HIV-1 and HIV-2 patients: A) Percentage of CD4 T cells expressing CD127^{lo/-} FOXP3⁺ in HIV-1 and HIV-2 patients with CD4 ≥ 500 cells/μl or <500 cells/μl. B) Absolute levels of CD4⁺CD127^{lo/-} FOXP3⁺ in HIV-1 and HIV-2 patients with CD4≥500 cells/μl or <500 cells/μl. The medians and interquartile ranges are displayed for each group. Data was analyzed using the Mann Whitney U test.

Similar results were obtained when percentage of $CD4^+CD25^+FOXP3^+$ was compared between HIV-1 and HIV-2 patients (1.51% (IQR: 0.9-2.1) versus 1.02% (IQR: 0.7-1.5), $p=0.0046$). There was very strong correlation between $CD4^+CD25^+FOXP3^+$ and $CD4^+CD127^{lo/-}FOXP3^+$ in both HIV-1 ($r = 0.9521$, $p=0.001$) and HIV-2 ($r=0.9321$, $p=0.001$) as well as between $CD4^+CD127^{lo/-}FOXP3^+$ and $CD4^+FOXP3^+$ (Figure 5.2).

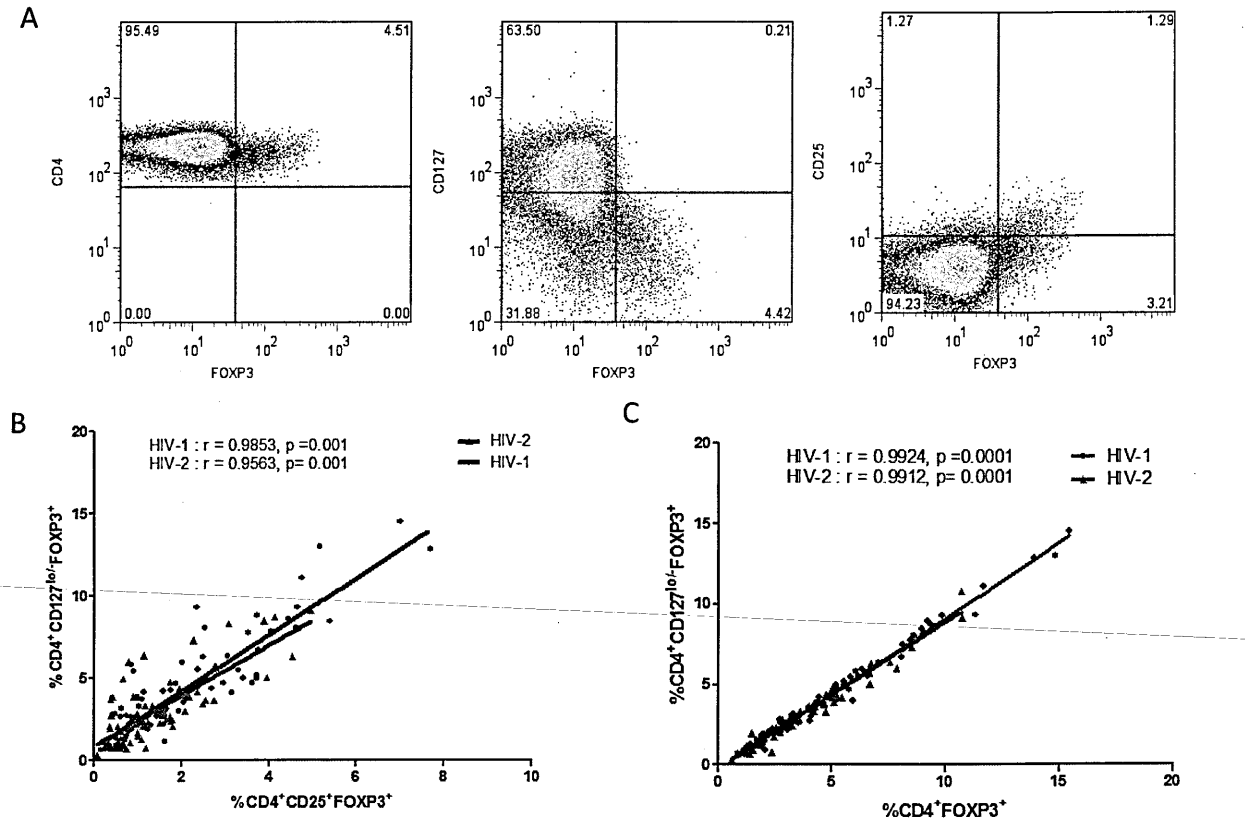


Figure 5.2: Phenotype of T regulatory cells in HIV-1 and HIV-2. A) Representative examples of whole blood staining of FOXP3 and CD4, FOXP3 and CD127, FOXP3 and CD25, gated on total CD4 T cells. B) Correlation between % $CD4^+CD25^+FOXP3^+$ and % $CD4^+CD127^{lo/-}FOXP3^+$ C) Correlation between % $CD4^+FOXP3^+$ and % $CD4^+CD127^{lo/-}FOXP3^+$. Correlations were assessed using the Spearman's rank test.

5.3.3. The proportion of CD4⁺CD127^{lo/-}FOXP3⁺ cells inversely correlates with CD4 T cell counts in HIV-1 and HIV-2.

The correlation between absolute CD4 T cell levels and percent or absolute Treg numbers was measured. In both HIV-1 and HIV-2 groups, there was a significant inverse correlation between percentage of Tregs and CD4 T cell numbers (HIV-1: $r = -0.3576$, $p = 0.0011$; HIV-2: $r = -0.3866$, $p = 0.0014$, Figure 5.3A). Similarly, the percentage of Tregs was inversely correlated with CD4 percentage in HIV-1 ($r = -0.4168$, $p = 0.0001$) and HIV-2 ($r = -0.2779$, $p = 0.01$).

There was a positive correlation between the absolute Tregs count and CD4 T cell count in both HIV-1 ($r = 0.3314$, $p = 0.0015$) and HIV-2 infected patients ($r = 0.3938$, $p = 0.0007$) (Figure 5.3B).

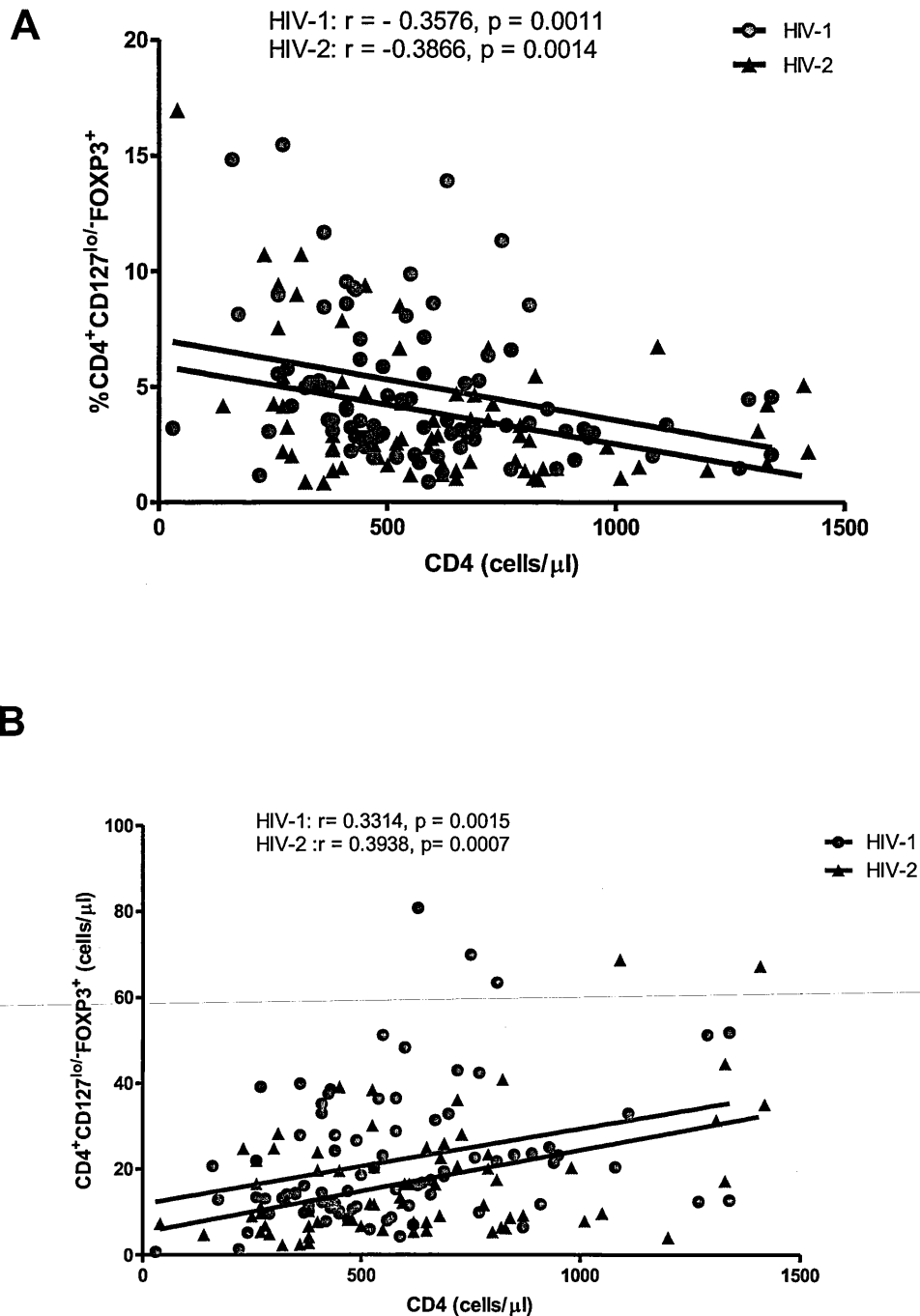


Figure 5.3: Correlation between percentage Tregs or absolute Treg count and absolute CD4 count A) Percentage of CD4 T cells expressing CD127^{lo/-}FOXP3⁺ inversely correlates with absolute CD4 T cell count in HIV-2 and HIV-1 (B) Absolute count of CD4⁺CD127^{lo/-}FOXP3⁺ cells positively correlates with absolute CD4 T cell count in HIV-2 and HIV-1 patients. Correlations were assessed using the Spearman Rank test.

5.3.4. Comparison of immune activation levels and the relationship with Tregs in HIV-1 and HIV-2

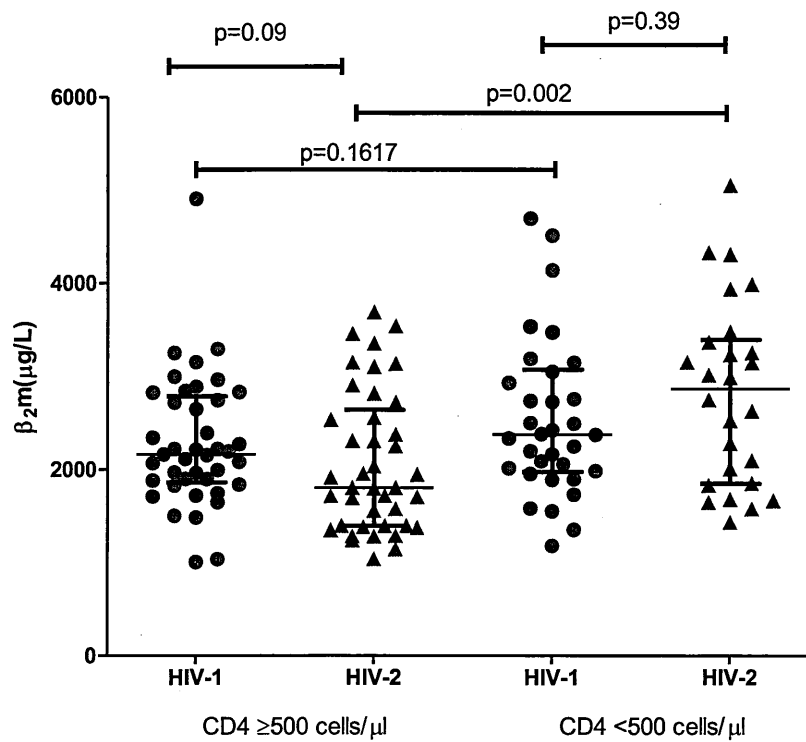
To determine the relationship between Tregs and immune activation, as measured by β -2 microglobulin (β 2m), their levels were correlated and compared in HIV-1 and HIV-2 patients with a high CD4 count (absolute CD4 \geq 500 cells/ μ l) or a low CD4 count ($<$ 500 cells/ μ l).

β 2m levels were significantly increased in patients with a low CD4 count compared to those with a high CD4 count in both HIV-1 (medians: 2180 μ g/L versus 2404 μ g/L, $p = 0.03$) and HIV-2 infection (medians: 2012 μ g/L versus 2687 μ g/L, $p = 0.0024$, Fig 5.4A). Indeed, there was a significant negative correlation between β 2m and absolute CD4 T cells levels in HIV-1 ($r = -0.2905$, $p = 0.015$) and HIV-2 infected patients ($r = 0.4879$, $p = 0.0001$).

Comparison of the β 2m levels between the HIV-1 and HIV-2 patients with a high CD4 count showed lower levels in HIV-2 patients but the difference did not reach statistical significance ($p = 0.08$). The levels were similar in HIV-1 and HIV-2 patients with a low CD4 counts ($p = 0.39$).

The percentage Tregs showed a weak positive correlation with β 2m levels in HIV-2 ($r = 0.3581$, $p = 0.0326$), while there was no correlation observed between β 2m levels with Tregs in the HIV-1 group ($r = 0.1923$, $p = 0.1595$, Figure 5.4B).

A



B

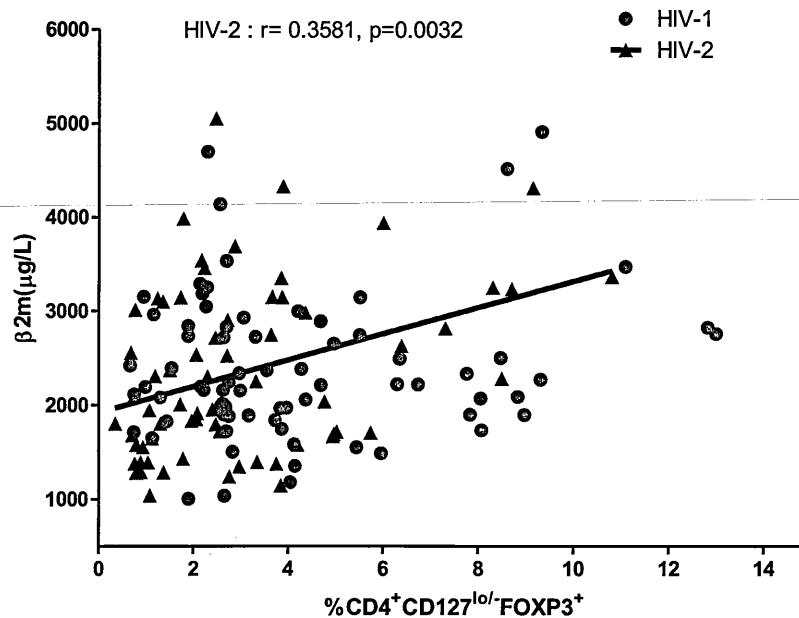


Figure 5.4: Comparison of immune activation in patients with HIV-1 and HIV-2 infection:

A) Comparison of β_2m levels in HIV-1 and HIV-2 infection stratified by CD4 counts (≥ 500 and < 500 cells/ μl); Data was analyzed using the Mann-Whitney test B) Correlation between β_2m levels and percentage Tregs in HIV-1 and HIV-2 infected patients. Correlation was assessed using Spearmans Rank test.

Plasma levels of $\beta 2m$ were positively correlated to viral load in both HIV-1 ($r = 0.4414$, $p = 0.007$) and HIV-2 ($r = 0.3903$, $p = 0.0014$) patients, (Figure 5.5A). However the percentage Tregs showed a moderate positive correlation with viral load (Fig 5.5B, $r = 0.4850$, $p = 0.0001$) in HIV-2 patients, while there was no correlation between Tregs and viral load in HIV-1 patients.

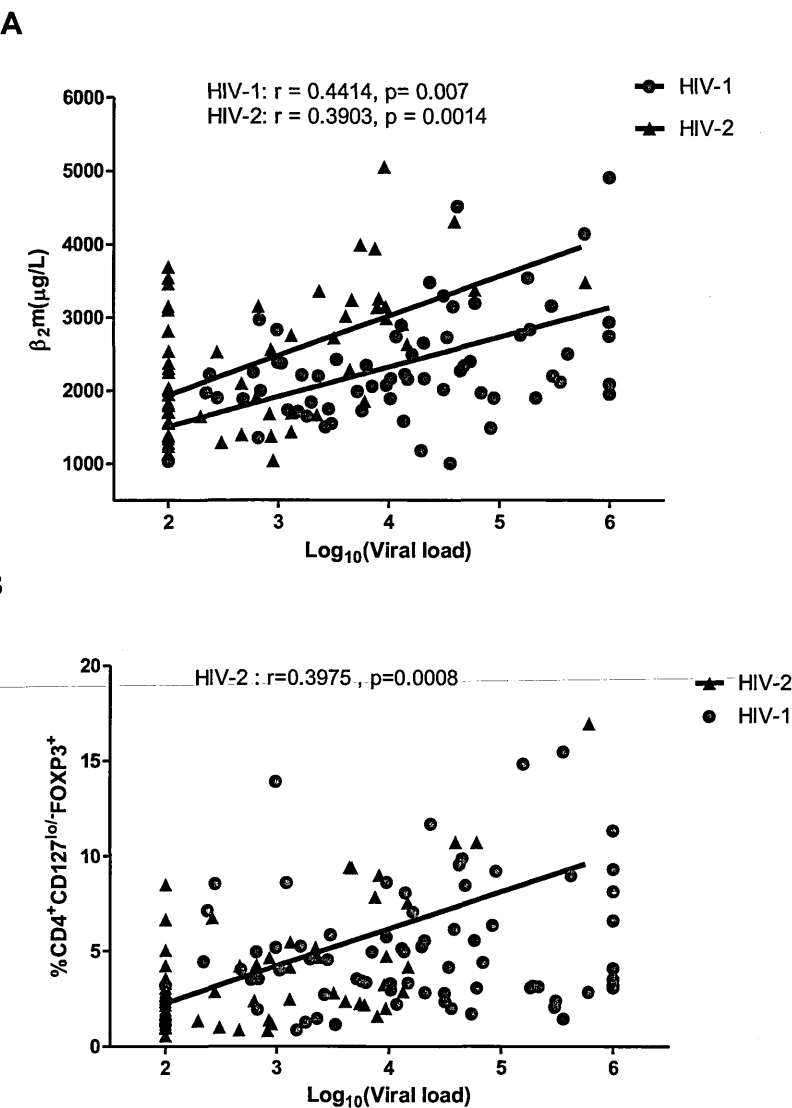


Figure 5.5: Relationship between viral load, immune activation and Treg percentage. A) Correlation of viral load with plasma level of $\beta 2m$ in HIV-1 and HIV-2 infected patients; B) Correlation between viral load and $\text{CD4}^+ \text{CD127}^{\text{lo/-}} \text{FOXP3}^+$ cells in HIV-1 and HIV-2 infected patients. Correlations were assessed using the Spearman Rank test.

5.4. Discussion:

HIV-2 infection predominantly results in long term non-progression and a greater proportion of infected individuals maintain high CD4 T cell counts and undetectable viral loads compared to those with HIV-1 infection. Immune activation is however a feature of both HIV-1 and HIV-2 infection and has been shown to predict progression to AIDS in both infections. It is well established that Tregs can modulate systemic and HIV specific immune responses though there is divided opinion on whether the suppressive activity of these cells is beneficial or detrimental in relation to HIV disease progression. In this study, Tregs were enumerated in asymptomatic and progressive HIV-2 and HIV-1 infected patients and correlated with markers of disease progression (immune activation and viral loads). The results demonstrate that asymptomatic HIV-2 patients have lower percentages of Tregs compared to asymptomatic HIV-1 patients or to HIV-2 patients with low CD4 T cell counts. However, though asymptomatic HIV-2 patients had lower absolute Tregs compared to patients with asymptomatic HIV-1 infections they were higher than HIV-2 patients with low CD4 counts. Furthermore, Tregs were positively correlated with immune activation level or viral load in HIV-2 but not in HIV-1 infected patients. Importantly these conclusions did not change when we defined Tregs as either $CD4^+CD127^{lo/-}FOXP3^+$ or $CD4^+CD25^+FOXP3^+$ since it was shown that two measurements were highly correlated (Figure 5.2B).

The controversy over Tregs in HIV infection is in large part due to definition of the cellular phenotype, differences in methodologies and differences in patients with regard to level of disease progression. In Chapter 4, it was shown that enumeration of Treg using flow cytometry does not correlate with FOXP3 transcript levels. Thus, Tregs were enumerated by flow cytometry using freshly taken whole blood to minimize any effects of *in vitro* manipulation and selecting $CD4^+CD127^{lo/-}FOXP3^+$ as appropriate markers for Treg

identification. Furthermore, HIV-1 and HIV-2 patients were matched by CD4 T cell counts which permitted a more detailed analysis between and within the respective infections. This study is the most comprehensive comparison of Tregs in HIV-1 and HIV-2 infected subjects to date. The only other study that compared Tregs between HIV-1 and HIV-2 showed no differences between the two infections (Foxall *et al.*, 2008). However, it is important to note that this study measured mRNA levels of FOXP3 as a surrogate for total levels of Tregs. Also, the smaller size of the study meant that they were unable to categorize patients by CD4 count and thus did not differentiate between asymptomatic and progressing HIV-2 infection.

Long term non-progression is a feature of the majority of HIV-2 infections. While this is rare in HIV-1 infections, a small proportion of HIV-1 infected patients (elite controllers) maintain low viral loads and high CD4 T cell counts similar to those patients with HIV-2 infection. Recent studies (that used a similar phenotype to measure Tregs by flow cytometry) showed that HIV-1 elite controllers and long term non-progressors had a lower percentage of Tregs compared with HIV-1 progressors (Schulze *et al.*, 2011, Hunt *et al.*, 2011). These studies support our findings which suggest that low Tregs are a feature of non-progressive HIV disease.

While it was clear that Tregs were significantly lower in HIV-2 asymptomatic infection compared to HIV-1, the dynamics of Tregs are similar in both infections. The percentage of Tregs increased as CD4 T cell counts declined, while the absolute numbers decreased. This suggests that Tregs are depleted during the course of HIV infection but do so at a different rate than other CD4 cells. More recent studies of patients with HIV-1 infection that have used flow cytometry and the FOXP3 marker to estimate the percentage and absolute numbers of Tregs have shown a similar relationship with CD4 T cell counts (Cao

et al., 2009, Bi *et al.*, 2009, Schulze zur Wiesch *et al.*, 2011). The relative increase in Tregs in asymptomatic HIV-1 infection compared to asymptomatic HIV-2 infection may be due to increased levels of immune activation (possibly due to higher levels of viral replication). Increased immune activation may lead to increased thymic output of Tregs or peripheral conversion of conventional CD4 T cells to Tregs as demonstrated previously (Walker *et al.*, 2003, Chen *et al.*, 2003). However it is evident that Tregs can themselves be infected and depleted by HIV-1 (Oswald-Richter *et al* 2004). A more recent study showed that HIV-1 CXCR4 tropic viruses more efficiently infected Tregs compared to CCR5 tropic viruses (Moreno-Fernandez *et al.*, 2009). Hence, preferential depletion of non-Tregs may also contribute to the percentage increase seen in Tregs at low CD4 T cell counts. Whether the HIV-2 virus shows similar cellular tropism remains to be studied.

Immune activation is a key factor in determining progression to disease in both HIV-1 and HIV-2. Viral replication damages the host gastrointestinal barriers leading to microbial translocation which contributes to increased immune activation in HIV-1 (Brenchley *et al.*, 2006). In HIV-2 infection, it is evident that a similar phenomena occurs (Nowroozalizadeh *et al.*, 2010). In our study the levels of β -2 microglobulin (β -2m, a component of the HLA Class 1 molecule) were significantly raised in progressive HIV-1 and HIV-2 and positively correlated with viral loads. Furthermore, asymptomatic HIV-2 patients had lower levels of plasma β -2m compared to HIV-1 patients with similar CD4 T cell counts. The finding that Tregs positively correlated with immune activation and viral load in HIV-2 and not HIV-1 infection suggests that in HIV-2 infection there may be a better balance between regulatory and effector responses. This balance may contribute to maintenance of HIV specific responses and is explored in Chapter 6.

In summary, the results from this chapter show that asymptomatic patients with HIV-2 infections have significantly lower Tregs compared to patients with HIV-1 infection and to those with progressive HIV-2 infection. Furthermore, these HIV-2 infected patients also displayed lower levels of systemic immune activation suggesting that low Tregs levels are a marker of non-progression in HIV infection.

Chapter 6: Functional characterization of effector responses and immune activation levels in HIV-1 and HIV-2 asymptomatic patients

6.1 Introduction

While a large proportion of HIV-2 infected individuals behave as long term non-progressors, it is estimated that this only occurs in less than one percent of HIV-1 infections (Okulicz *et al.*, 2009, Lambotte *et al.*, 2005). These dichotomous outcomes could be due to host genetics, viral factors or the immune response against the respective viruses. Hence HIV-2 infection presents an intriguing contrast to HIV-1 infection and presents an opportunity to understand the factors that lead to successful viral control. These attributes would be useful in developing an efficacious HIV vaccine. Several studies have provided evidence that T cells that recognize epitopes in the Gag region of HIV-1 and HIV-2 are likely to play a role in suppression of viral replication (Leligdowicz *et al.*, 2007, Betts *et al.*, 2007). In addition, the degree of polyfunctionality of these cells manifest by the ability to produce different effector cytokines such as IL-2, TNF- α or IFN- γ is inversely correlated with viral load level in HIV-1 infection (Betts *et al.*, 2006). Direct comparison of HIV-1 and HIV-2 infections suggest that polyfunctional T cells are better preserved in HIV-2 infected patients relative to HIV-1 (Duvall *et al* 2008). Collectively these studies suggest that these responses are important in controlling viral replication. However, in the recent Merck trial although the STEP HIV-1 vaccine elicited high levels of polyfunctional responses (IFN γ and TNF- α , but not IL-2) it failed to protect against infection (McElrath *et al* 2008). Examination of the possible reasons for the failure of this vaccine suggested that increased immune activation elicited by the Adenovirus vector 5 (Ad5) used in the vaccine construct may have contributed to higher infection rates in the vaccinated group (Buchbinder *et al.*, 2008). This has forced the scientific community to re-examine the correlates of protection during HIV infection and decipher the relationship between immune activation and HIV specific immune responses.

It is evident that the immune activation is raised in both HIV-1 and HIV-2 infections relative to uninfected healthy individuals and the degree of activation has been shown to accurately predict progression to AIDS (Giorgi *et al.*, 1999, Hazenberg *et al.*, 2003, Jaffar *et al.*, 2005, Michel *et al.*, 2000). Whether HIV specific immune responses influence generalized immune activation (or vice versa) is unclear. In HIV-2 infections this relationship remains vague, with one study showing no correlation between HIV-2 Gag responses and immune activation (Lelgiewicz *et al* 2010), while another showed a positive correlation between activated CD4 T cells and CD4 Gag responses (Foxall *et al* 2008).

T regulatory cells (Tregs) are a subset of CD4 T cells that can curtail activation of immune cells. While multiple lineages of these cells have been described, CD4 T cells expressing the transcription factor FOXP3 has received the greatest attention. The role of Tregs during HIV infections remains controversial since it is as yet unclear whether their activity is helpful or deleterious. Many studies have shown that *In vitro* Tregs can suppress IFN- γ production and T cell proliferation in HIV infected individuals, which suggested that Treg activity may hamper viral control. However, other accounts suggest that Treg activity was beneficial in HIV-1 infection as it inversely correlated with systemic immune activation. The results in chapter 5 showed that patients with high CD4 T cell counts had the lowest level of Tregs and in addition, HIV-2 patients with high CD4 counts had significantly lower Tregs compared to CD4 matched HIV patients. The aim of the work reported in this chapter is to determine the relationship between immune activation and specific immune responses in HIV-1 and HIV-2 patients with low Treg frequencies.

6.2 Methods

6.2.1 Patients:

HIV-1 and HIV-2 patients found to have low Tregs levels (Chapter 5) were recalled and provided an additional aliquot of blood. The viral loads and CD4 levels were tested for all patients. PBMC were obtained from all patient samples and used to determine Gag specific immune responses as well as levels of activated CD4 and CD8 T cells.

As before, patients that presented with evidence of concurrent infections such as malaria or tuberculosis were excluded. Table 6.1 summarizes the characteristics of patients recruited to the study.

HIV type	Age(yrs)	% Female	CD4%	CD4 (cells/ μ l)	Log Viral load (copies/ml)	Years since diagnosis
HIV-1 (n=23)	35 (30-53)	73	29 (23-32)	620 (530-780)	4.13 (3.22-4.34)	2 (2-4)
HIV-2 (n= 23)	45 (37-51)	76	36 (30-43)	790 (600-1010)	2.00 (2.00-2.95)	8 (3-15)

Table 6.1: Characteristics of asymptomatic HIV-1 and HIV-2 subjects. All values displayed are median values and the interquartile ranges are displayed in brackets. Bold values indicate a statistically significant difference between the groups at $p < 0.05$.

6.2.2 Intracellular cytokine assays

The levels of HIV-1 and HIV-2 Gag specific CD4 and CD8 T cells were evaluated using overnight stimulation followed by intracellular cytokine staining as described in Chapter 3, section 3.8.

6.2.3. Enumeration of activated CD4⁺ and CD8⁺ T cells

Activated T cells were defined as co-expression of CD38 and HLA-DR and measured as described in Chapter 3, section 3.8.

6.2.4. Depletion assays

To assess whether Tregs inhibited HIV-2 specific and SEB induced responses CD25⁺ cells were depleted and stimulated in parallel with undepleted samples as described in Chapter 3, section 3.9

6.2.5. Statistics

Data was analyzed using Stata software Version 11(Stata Corp, USA) and Graphs were prepared using Prism (version 5). Unpaired data was analyzed using the non-parametric Wilcoxon rank sum test; while paired data was analyzed using the Wilcoxon matched pairs test. Correlations were tested using Spearman's rank correlation co-efficient for non-normally distributed data. Statistical test results were considered significant if $p < 0.05$.

6.3 Results:

6.3.1 Characteristics of participants

The majority of study participants were predominantly female in both HIV-1 and HIV-2 infected groups. HIV-2 study subjects had a median age of 45 years which was ten years greater than that of median age of HIV-1 subjects. The median CD4 level was significantly higher and viral load significantly lower in the HIV-2 group. Seventy three percent of HIV-2 subjects had an undetectable viral load and the median time since diagnosis was eight years. The HIV-1 patients were more recently diagnosed with median time since diagnosis of two years (Table 6.1).

6.3.1 HIV-2 subjects elicit higher polyfunctional responses to Gag antigen

The percentages of CD4 and CD8 T cells expressing IL-2, IFN- γ and CD107a after HIV specific or SEB stimulation were determined and background levels (CD28/CD49d control) were subtracted (Figure 6.1).

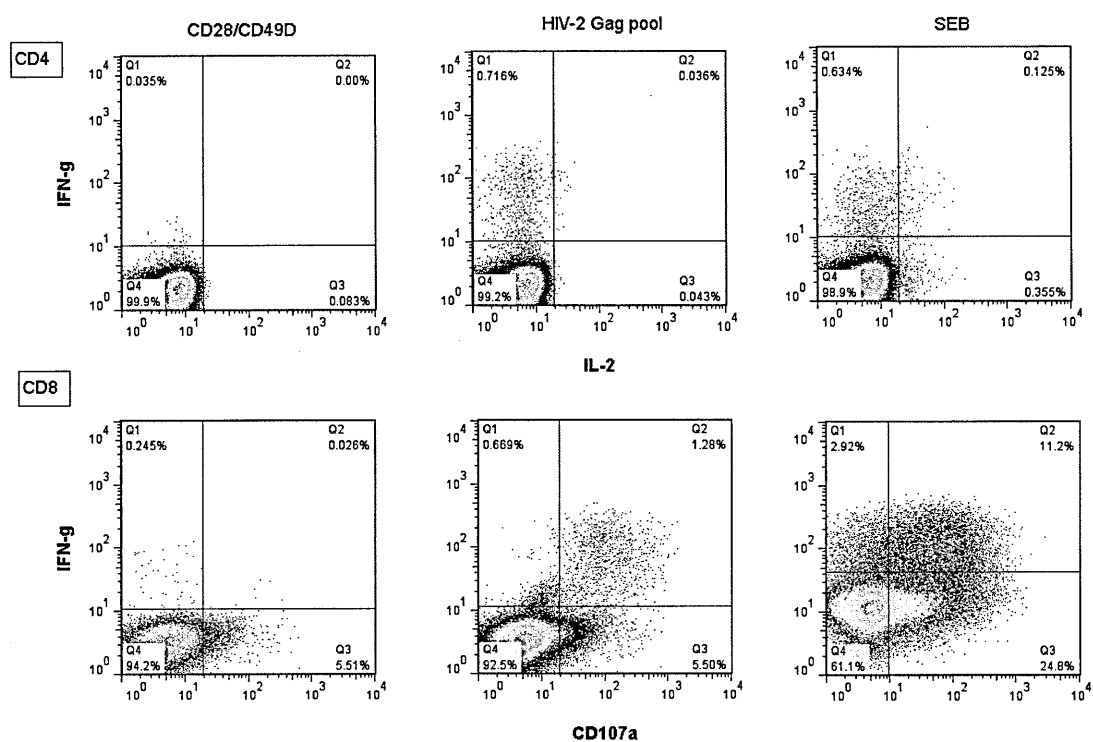


Figure 6.1: Intracellular cytokine responses after overnight stimulation. A) CD4 cells expressing IL-2, IFN- γ B) CD8 T cells expressing IFN- γ and CD107a Gag or SEB.

CD4 T cells secreted IL-2, IFN- γ or both after HIV Gag or SEB stimulation. CD8 T cells responding to HIV Gag peptides up regulated IFN- γ and co-expressed CD107a, a marker that is expressed on cells that are degranulating.

CD4⁺ and CD8⁺ T cells producing IFN- γ after HIV Gag stimulation were observed in both HIV-1 and HIV-2 subjects (Table 6.2). The percentage of positive CD4 and CD8 T cells in the unstimulated control were subtracted from those in the peptide and SEB stimulated cultures. There was no difference in the median levels of IFN- γ secreting CD4 (HIV-1:0.129%, HIV-2 : 0.139% ,p =0.8802) or CD8 (HIV-1: 0.21%, HIV-2: 0.26%, p=0.6130) T cells between HIV-1 and HIV-2 in the unstimulated controls. A positive response was defined as that which was above the background value.

All HIV-2 patients tested showed a positive response, by either CD4⁺ or CD8⁺ T cells to Gag peptides compared with 78% in HIV-1 infected individuals (p=0.001). In addition, a higher proportion of HIV-2 subjects (75%) had both a CD4 and CD8 T cell response compared with only 21% in the HIV-1 infected group (p=0.01). PBMC from both HIV-1 and HIV-2 showed a CD4 and CD8 T cell responses after stimulation with the positive control SEB.

HIV type	Total responders	CD4 responders only	CD8 responders only	CD4+CD8+ responders
HIV-1	18/23 (78%)	8/23 (33%)	4/23 (17%)	5/23 (21%)
HIV-2	21/21 (100%)	3/21 (15%)	2/21 (10%)	16/21 (75%)

Table 6.2: Summary of HIV-1 and HIV-2 patients showing a positive Gag response by T cell subsets

After stimulation with gag peptides the percentage of Gag specific CD4⁺IFN γ ⁺ cells (median: HIV-1: 0.001, HIV-2: 0.1, p=0.001), CD4⁺IFN γ ⁺ IL-2⁺ cells (median: HIV-1:0, HIV-2: 0.02, p=0.0001) and CD4⁺IL-2⁺ cells (median: HIV-1:0, HIV-2: 0.02, p=0.003) were significantly higher in the HIV-2 infected patients compared to the HIV-1 patients (Figure

6.2A). Similarly, the levels of CD8⁺IFN- γ ⁺ staining cells were significantly higher in the HIV-2 group compared to HIV-1 after Gag stimulation (p=0.0035, Figure 6.2B)

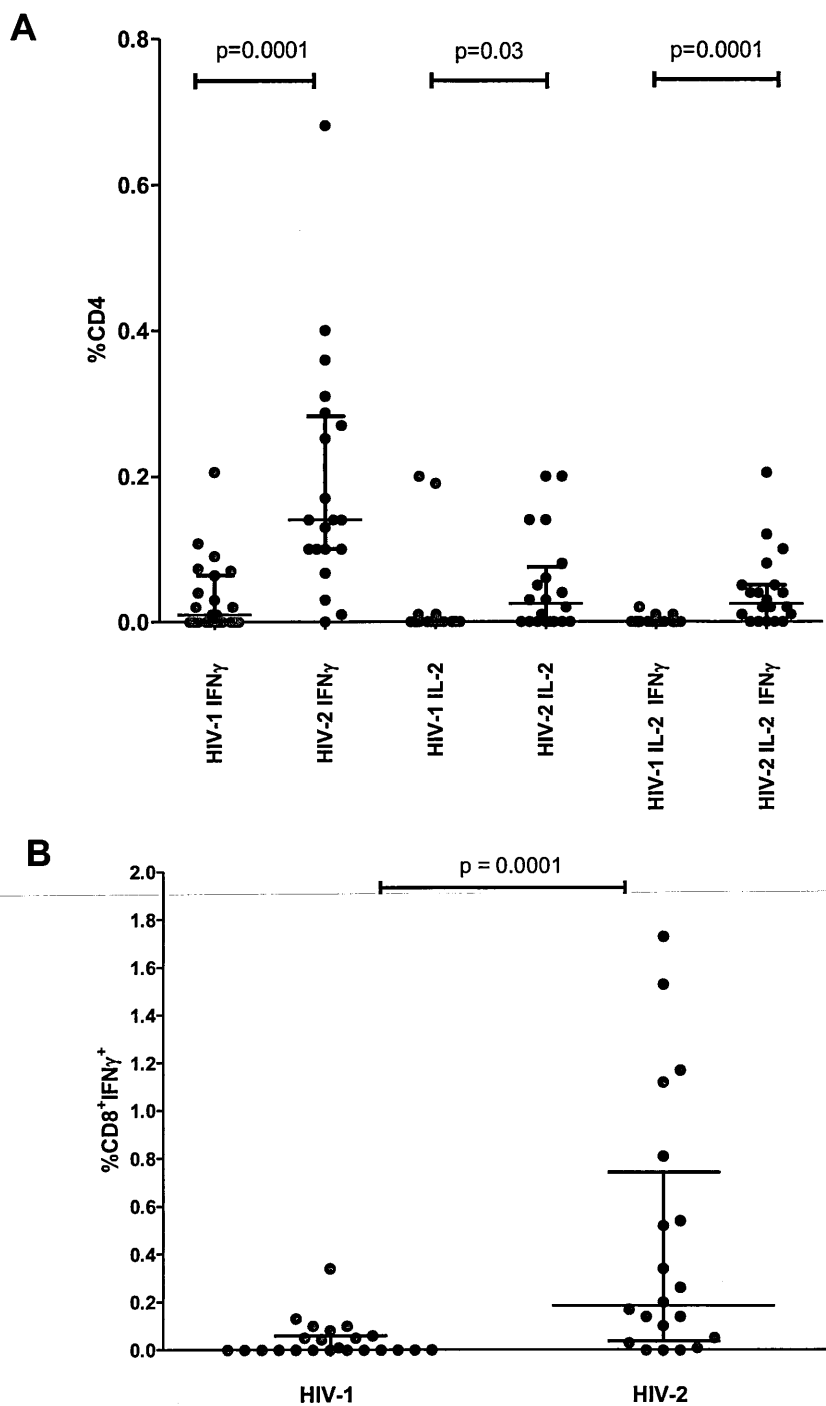


Figure 6.2: Comparison of Gag specific responses in HIV-1 and HIV-2. A) CD4 T cell and B) CD8 T cell responses in HIV-1 and HIV-2 subjects. The Mann-Whitney U test was used to measure differences between groups.

6.3.2. Comparison of T cell activation between HIV-1 and HIV-2 patients

Immune activation is a central feature of both HIV-1 and HIV-2 infections. To determine the immune activation levels within the T cell compartment in the study samples co-expression of cyclic ADP ribose hydrolase (CD38) and human leukocyte antigen (HLA-DR) on CD4 and CD8 T cells was measured using six-colour flow cytometry as described in the methods sections. Activated cells were defined as those co-expressing HLA-DR and CD38 (Figure 6.3A).

The median percentage of CD8⁺CD38⁺HLA-DR⁺ were significantly lower in HIV-2 patients compared to HIV-1 subjects (HIV-1: 9.48% (IQR: 7.17-15.48), HIV-2: 5.3% (IQR: 4.38-5.76); $p=0.002$). Similarly, the proportion of CD4⁺CD38⁺HLA-DR⁺ cells in the HIV-1 patients (median: 2.6% (IQR: 1.92-4.53%)) was significantly higher than the HIV-2 patients (median 1.67 % (IQR: 1.19-1.81), $p = 0.001$, Figure 6.3). The median percentage of CD4 T cells expressing either CD38 (HIV-1:41.7 HIV-2:36.7, $p=0.1423$) or HLA-DR (HIV-1: HIV-2:, $p=0.4484$) only, were similar between the two groups of patients. Similarly, the median percentage of CD8 T cells expressing either CD38 (HIV-1:31.26 HIV-2:27.82, $p=0.6400$) or HLA-DR (HIV-1:6.96 HIV-2: 4.825, $p=0.2373$) only, were similar (data not shown). The percentage of CD4⁺CD38⁺HLA-DR⁺ cells was positively and significantly correlated with CD8⁺CD38⁺HLA-DR⁺ in HIV-1 infected patients ($r=0.8248$, $p=0.001$) and HIV-2 patients ($r=0.6529$, $p=0.002$, figure 6.3B and C).

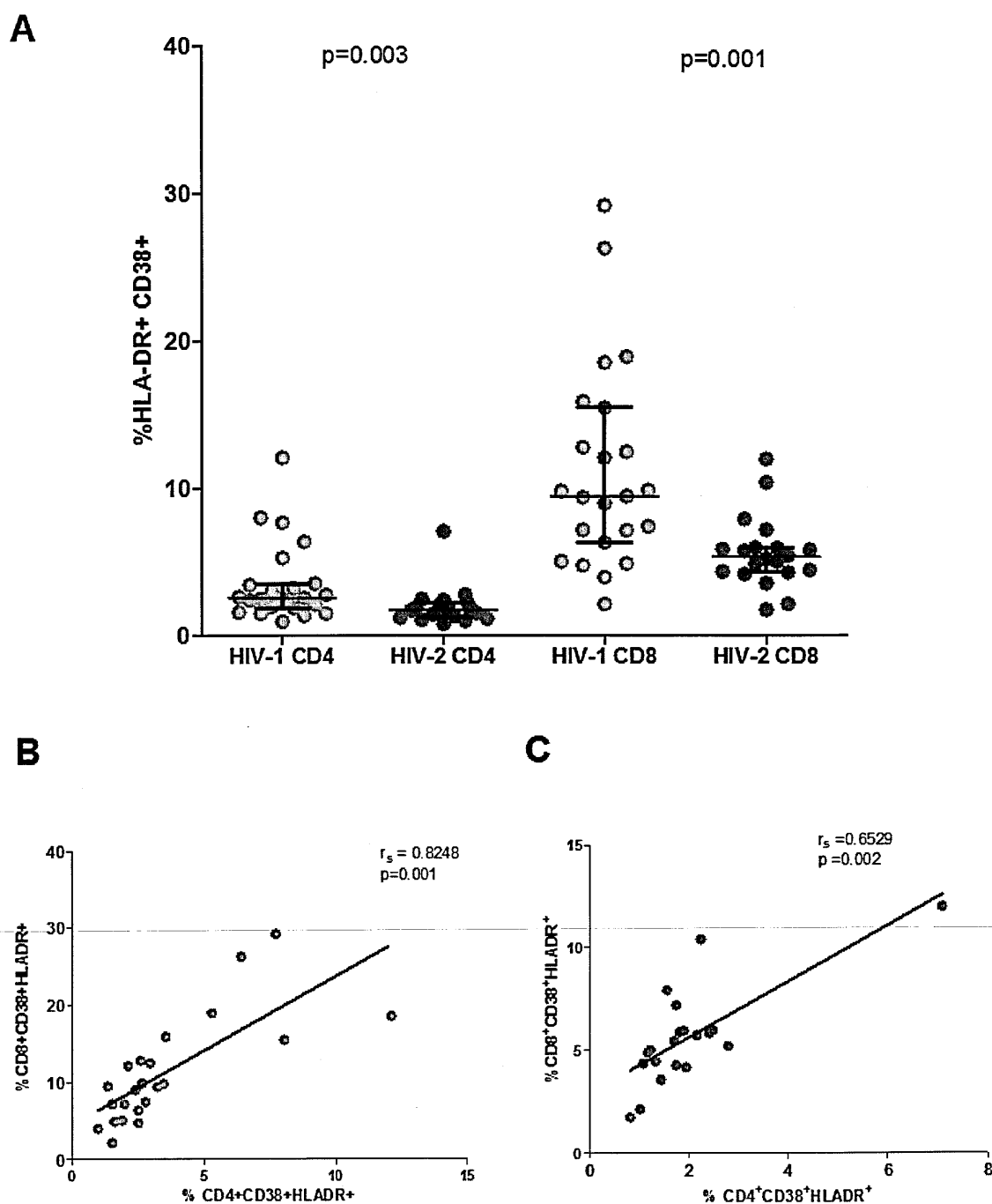
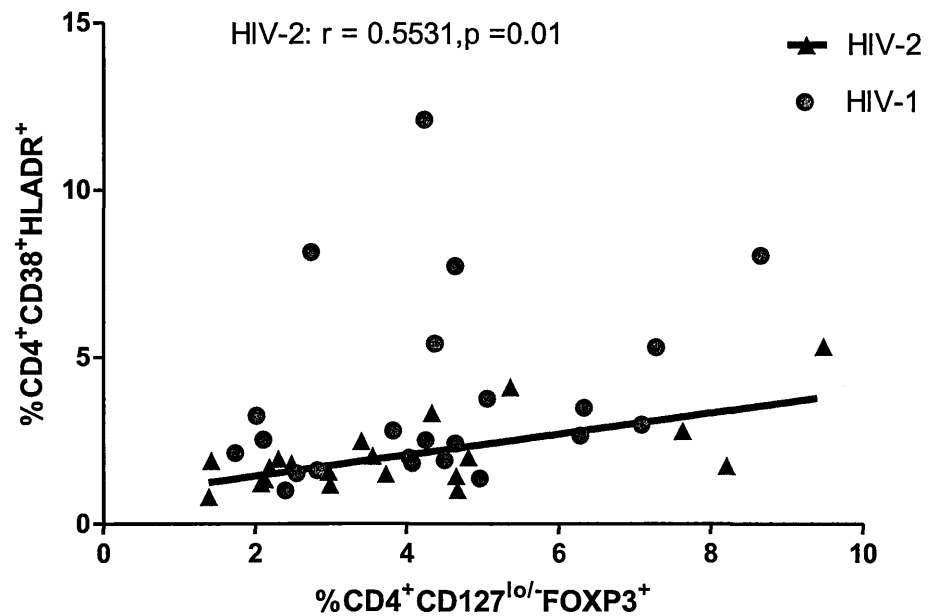


Figure 6.3: Comparison of T cell activation in HIV-1 and HIV-2 asymptomatic patients. A) Comparison of $CD4^+CD38^+HLA-DR^+$ and $CD8^+CD38^+HLA-DR^+$ cells in HIV-1 and HIV-2 patients. Horizontal bars represent the median values and interquartile ranges are indicated. Correlation of $CD4^+CD38^+HLA-DR^+$ and $CD8^+CD38^+HLA-DR^+$ in HIV-1 (B) and HIV-2(C). The r_s values indicate the correlation statistic using the spearman rank test.

6.3.3. Relationship of activated T cells and Tregs in HIV-1 and HIV-2

The percentage of CD4⁺CD38⁺HLA-DR⁺ or CD8⁺CD38⁺HLA-DR⁺ T cells was correlated with those of Tregs as defined by CD4⁺CD127^{lo/-}FOXP3⁺ markers in HIV-1 and HIV-2 infected patients. The percentage of CD4⁺CD38⁺HLA-DR⁺ cells showed a modest positive correlation with percentage Tregs in HIV-2 infected subjects ($r = 0.5531$, $p=0.01$, Figure 6.4A). Similarly the percentage of CD8⁺CD38⁺HLA-DR⁺ was also positively correlated with the percentage Tregs in HIV-2 infected patients ($r=0.4736$, $p=0.03$, Figure 6.4B). There was no correlation observed between Tregs and CD4⁺CD38⁺HLA-DR⁺ or CD8⁺CD38⁺HLA-DR⁺ in the HIV-1 patients.

A



B

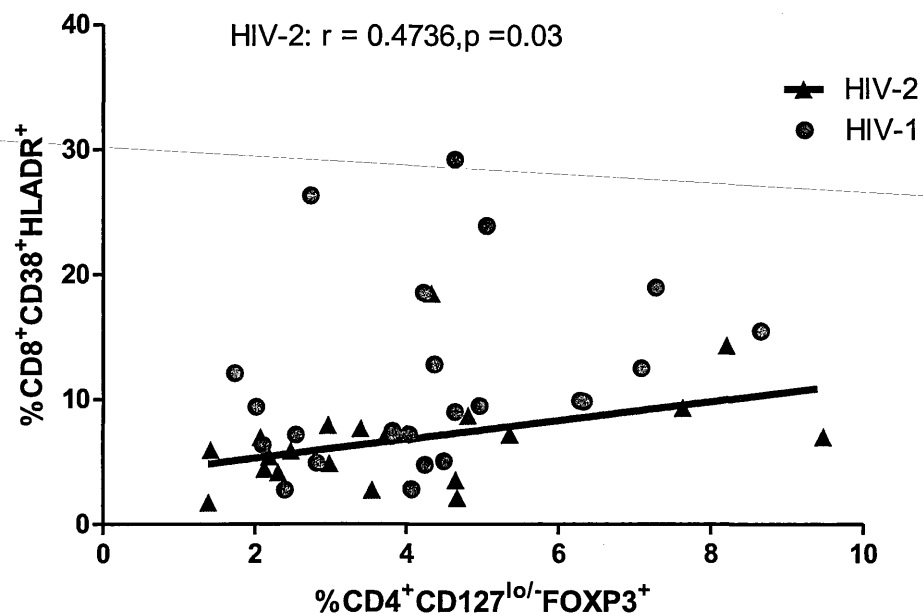


Figure 6.4: Relationship between Tregs and activated T cells. A) Correlation of percentage CD4⁺CD127^{lo/-}FOXP3⁺ and CD4⁺CD38⁺HLA-DR⁺ cells in HIV-1 and HIV-2 infected patients B) Correlation of percentage CD4⁺CD127^{lo/-}FOXP3⁺ and CD8⁺CD38⁺HLA-DR cells in HIV-1 and HIV-2 infected patients. Correlation was assessed using the spearman rank test.

6.3.4 Relationship of immune activation and HIV specific immune responses in HIV-2 infection

Having determined the levels of immune activation and HIV specific immune responses in the HIV-1 and HIV-2 patients, we aimed to determine whether the two were correlated. No correlation was observed between the percentage of CD4⁺CD38⁺HLA-DR⁺ and CD4⁺IFN γ ⁺ cells in HIV-2 ($r=-0.3289$, $p=0.1258$) or HIV-1 infection ($r=0.2451$, $p=0.2123$). No correlation was observed between percentage CD8⁺CD38⁺HLA-DR⁺ and CD8⁺IFN γ ⁺ cells in either the HIV-1 ($r= -0.3745$, $p=0.09$) or HIV-2 ($r=-0.2283$, $p=0.1563$) infected patients (Figure 6.4).

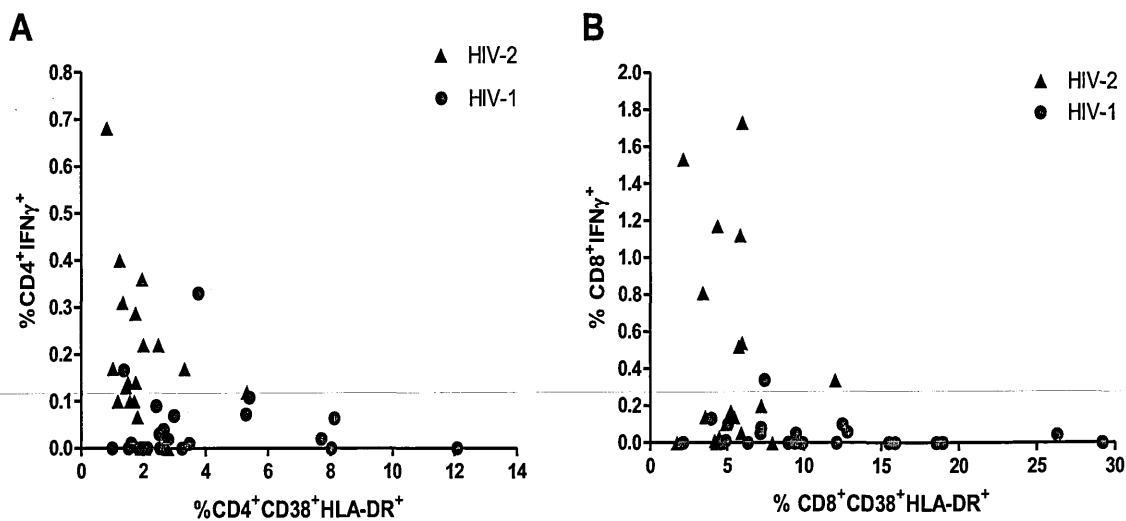


Figure 6.5: Correlation between immune activation and HIV Gag specific IFN- γ responses. A) CD4⁺IFN- γ ⁺ responses and percentage CD4⁺CD38⁺HLADR⁺ cells B) CD8⁺IFN- γ ⁺ responses and CD8⁺CD38⁺HLADR⁺ cells in HIV-1 and HIV-2 infected patients.

6.3.5 HIV-2 specific Tregs inhibit HIV specific Gag responses

Having determined the magnitude of responses in CD4 and CD8 T cells we sought to ascertain whether Tregs from HIV-2 infected subjects were functional. PBMCs from seven HIV-2 patients that had previously responded to a pool of HIV-2 Gag peptides were used for the Treg depletion and ICS assays.

The characteristics of the patients selected for the depletion experiments are summarized in Table 6.3.

Donor	Subset of cells responding to gag	CD4%	CD4 (cells/ μ L)	Viral load(copies/mL)	yrs since diagnosis(years)
1	CD4/CD8	41	980	673	17
2	CD4/CD8	31	780	100	16
3	CD4	35	1040	100	15
4	CD8	52	1410	100	20
5	CD4	26	520	100	10
6	CD4/CD8	42	1090	290	12
7	CD4	31	790	100	10

Table 6.3: Characteristics of patients used for depletion experiments

Analysis of the CD25⁺ fraction obtained from PBMCs showed that >95% of these cells expressed FOXP3 and they were all CD4⁺. Depletion resulted in a median 42% (range= 30 -74%) reduction in the levels of CD4⁺FOXP3⁺ cells in the PBMCs (Figure 6.6A) and a 99% reduction of CD4⁺CD25⁺ cells. Removal of Tregs resulted in a modest increase in percentage of IFN- γ from CD4 T cells (Figure 6.6C, p=0.06). Of note, Treg depletion did not increase the levels of CD4⁺IL-2⁺ cells in any of the donors. Depletion of CD25⁺ cells

resulted in increased CD8 T cell responses after Gag stimulation from all patients (Figure 6.6 D, $p=0.01$). These were predominantly polyfunctional in nature as the CD8 T cells expressed both IFN- γ and showed evidence of degranulation as measured by CD107a expression (Figure 6.6B) .

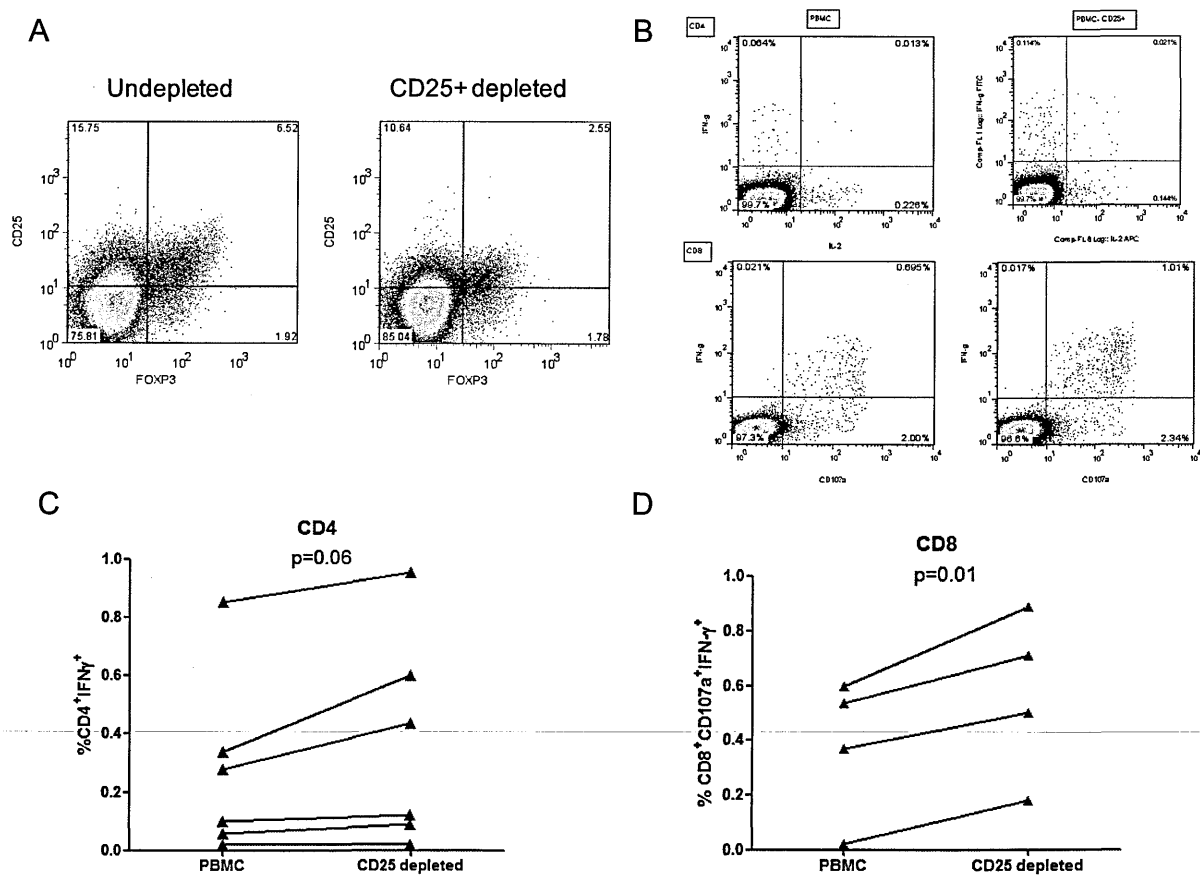


Figure 6.6: Effects of Treg depletion on percentage of IFN- γ secreting cells after Gag and SEB stimulations. A) Representative plot of undepleted and CD25 depleted CD4 T cells B) Comparative plots of CD8 and CD4 T cell responses from whole PBMCs and CD25 depleted samples C) Comparison of percentage IFN- γ producing CD4 T cells in undepleted and CD25 depleted PBMC after HIV-2 Gag stimulation D) Comparison of CD8 T cells co-expressing CD107a and IFN- γ in undepleted and CD25 depleted PBMC after HIV-2 Gag stimulation

6.4. Discussion

In Chapter 5, it was demonstrated that HIV-2 asymptomatic patients harboured significantly lower Treg levels than HIV-1 patients and these correlated with viral load and immune activation in the HIV-2 patients only, suggesting more efficient immune control than those in HIV-1 infection. To explore the relationship between immune activation and HIV specific immune responses, asymptomatic HIV-1 and HIV-2 patients with preserved CD4 T cell counts (>500 cells/ μ l) and low Treg levels were selected. The majority of HIV-2 patients displayed characteristics of long-term non progression as determined by a high CD4 T cell count, undetectable viral load and a median time since diagnosis of at least eight years. In contrast, HIV-1 patients were more recently diagnosed and despite high CD4 T cell counts, had a median viral load two logs higher than that of HIV-2 patients. This suggests that while the HIV-2 patients are true non-progressors, the majority of HIV-1 patients in this study will eventually progress to AIDS.

It is well established that immune function is better preserved in HIV-2 asymptomatic patients than in HIV-1 patients. Studies have demonstrated that the magnitude, breadth and polyfunctionality of T cell responses are greater in HIV-2 patients than HIV-1 (Duvall *et al.*, 2006, 2008, Lopes *et al.*, 2003). Furthermore, the magnitude of T cell responses has been shown to inversely correlate with viral load, suggesting these cells are effective in controlling viral replication (Jaye *et al.*, 2004, Leligdowicz *et al.*, 2007). In support of these findings, HIV-2 patients displayed greater magnitude and polyfunctionality of both CD4 and CD8 T cells compared to HIV-1 subjects in this study. Previous studies measuring CD8 T cell responses and total PBMC responses did not show differences between IFN- γ levels between HIV-1 and HIV-2 infected patients (Jaye *et al.*, 2004, Ondondo *et al.*, 2008). The varying results maybe as a result of differences in methodologies such as the use of ELispots as opposed to Intracellular Cytokine staining and flow cytometry.

A high level of immune activation has been shown to be associated with disease progression in both HIV-1 and HIV-2. Immune activation probably increases the availability of target cells for infection as well as inducing greater replication of the virus in latent pools. In chapter 5, it was shown that immune activation was positively correlated with viral load in both infections and was marginally lower in asymptomatic HIV-2 infected subjects than in HIV-1 infected subjects. We now show that in asymptomatic HIV-2 patients had significantly lower activation of CD4 and CD8 T cell compared to HIV-1 patients. While a previous study had demonstrated lower activation of CD8 T cells in HIV-2 subjects compared to HIV-1 infected individuals, they failed to detect differences in the levels of activated CD4 T cells (Hanson *et al.*, 2005).

In Chapter 5 a positive correlation between Tregs and immune activation in HIV-2 patients but not HIV-1 patients was found. Now we show that Tregs are also positively correlated with activated CD4 and CD8 T cells in the HIV-2 group only. This suggests that in HIV-2 infection increasing immune activation is balanced by a proportional Treg response, while this balance is lost in HIV-1 infection.

The results show that the features of long-term nonprogression in HIV-2 are low levels of Tregs which control immune activation and preserve immune function. It is pertinent to compare the results here with similar studies conducted in the so called HIV-1 'elite controllers'. These patients maintain a low viral load and high CD4 count that is reminiscent of the majority of HIV-2 infections. A study by Owen *et al.*, (2010) showed that elite HIV-1 infected controllers had lower levels of activated T cells but similar levels of Tregs when compared to HIV-1 non-controllers. In another study HIV-1 controllers had high levels of activated T cells and low levels of Tregs compared to HIV-1 progressors (Hunt *et al.*, 2011). The latter study suggested that these low Treg levels allowed effective

adaptive responses but also higher systemic immune activation. The differences between the conclusions of these studies maybe due to the phenotype used to enumerate Tregs. While both studies used CD38 and HLA-DR co-expression to define activated cells, neither used FOXP3 as part of their signature to measure Tregs.

Treg activity may play a pivotal role in determining the outcome of infection for the suppressive capacity of these cells has been associated with favourable outcome in both HIV-1 and SIV infections (Kinter *et al.*, 2004, Karlsson *et al.*, 2011). Similarly, we found that Tregs were functional in HIV-2 infected patients and that removal of Tregs resulted in increased IFN- γ secretion from CD4 T cells and IFN- γ and degranulation of CD8 T cells. These findings suggest that Tregs do control HIV-2 specific immune responses and may impact on the control of viral replication.

In conclusion, the results from this chapter show that HIV-2 patients with the characteristics of long-term non progressors have low Treg levels and maintain high HIV-2 specific immune responses accompanied by low levels of activation in the CD4 and CD8 T cell compartment. The findings also reinforce the idea that a useful balance between Tregs and immune activation exists in HIV-2 infections, but is conspicuously absent in HIV-1 infections.

Chapter 7: Immune Reconstitution Inflammatory Syndrome (IRIS): delayed reconstitution of T regulatory cells?

7.1. Introduction

The Immune Reconstitution Inflammatory Syndrome (IRIS) results from an exuberant immune response against residual antigens (paradoxical IRIS) or against viable pathogens (unmasking IRIS) in HIV infected patients commencing antiretroviral therapy (Shelburne *et al.*, 2005, French *et al.*, 2004, Lawn *et al.*, 2005). While most cases of IRIS develop in response to *Mycobacteria*, a number of other pathogens (such as cytomegalovirus and cryptococcal infections) have also been associated with the development of IRIS (Murdoch *et al.*, 2007).

The reported incidence of IRIS varies and a recent meta analysis showed a range from 10% to 32% for all cause IRIS (pathogen unidentified) (Muller *et al.*, 2010). The peak incidence of IRIS occurs 2 to 8 weeks after ART initiation, and both paradoxical and unmasking IRIS are more common in patients with a low CD4 T cell count prior to starting ART (Valin *et al.*, 2010, Ratnam *et al.*, 2006). While IRIS often has a benign course, it can cause considerable morbidity and occasionally be fatal (Lawn *et al.*, 2009).

The etiology of IRIS remains enigmatic, including its immunological mechanism and predictors. Increased proliferation and production of IFN- γ from highly activated CD4 T cells towards tuberculin, and increased levels of KIR- $\gamma\delta$ + T cells were observed in TB- IRIS compared to non-IRIS ART controls (Bourgarit *et al.*, 2006 & 2009). A more recent study demonstrated that IRIS patients displayed higher levels of HLA-DR⁺, Ki-67⁺, PD-1⁺ and effector memory subsets of CD4 T cells compared to non-IRIS controls (Antonelli *et al.*, 2010). Similarly, using a model of *Mycobacterium avium* (*M. avium*) challenge in lymphopenic mice, Barber *et al.* (2010), demonstrated the onset of symptoms consistent with IRIS after injection of exogenous CD4 T cells. Together these studies suggest that aberrant activation of reconstituted CD4 T cells is the underlying cause of IRIS.

T regulatory cells (Tregs) are committed suppressors of the immune system. While multiple subtypes have been described, the best characterized remain the natural Tregs which originate from the thymus (Khattari *et al.*, 2003), but found to be also induced in the periphery. Tregs function by suppressing the secretion of cytokines and proliferative responses by a variety of immune cell types (Sakaguchi *et al.*, 2010) in response to microbial and self-antigens (Sakaguchi *et al.*, 2004, Suffia *et al.*, 2006). The role of Tregs in the development of IRIS remains contentious, as a recent study showed no differences in Treg levels between patients developing TB-IRIS and asymptomatic patients (Meintjes *et al.*, 2008) while another study found that Tregs from patients with IRIS due to *M. avium* were less suppressive than Tregs from non-IRIS ART controls (Seddiki *et al.*, 2009).

The clinical symptoms of IRIS are consistent with a dysregulated and exuberant immune response which suggests the absence of effective immune regulation. Using a prospective longitudinal study design we have focused on the dynamics of Tregs following the start of ART and tested the hypothesis that (all-cause) IRIS results from a relative delay in the reconstitution of FOXP3 positive T regulatory cells. In addition, we assessed whether baseline plasma cytokines could predict the occurrence of IRIS after initiation of ART. The results from this study argue against a significant role for Treg levels, for neither the proportion nor absolute number, were predictors of IRIS.

7.2. Materials and Methods:

7.2.1 Clinical

In a prospective observational cohort conducted at the MRC Laboratories in The Gambia, Seventy- one consecutive ART naïve adult African patients, who had a nadir CD4 count < 200 and were scheduled to commence ART were asked for informed consent prior to inclusion in the study. Fifty eight of these patients were singly infected with HIV-1, 9 with HIV-2 only, while 4 were dually infected with both HIV-1 and HIV-2. Patients were screened for TB at baseline by reviewing symptoms and chest radiographs. They were encouraged to return to the clinic anytime they developed new symptoms. On their scheduled visits at 2, 4, 8, 12, and 24 weeks post ART initiation, patients were asked specific questions about IRIS prior to seeing a physician. These included questions as to whether they had a fever, night sweats, cough, headache, nausea, abdominal pain, weakness, visual problems, skin lesions, or any swelling. The physicians assessed each patient's likelihood of having IRIS, and all cases were subsequently reviewed and classified as IRIS based on the criteria outlined by Haddow *et al.* (2009, see Appendix)

7.2.3 Laboratory

Patients had viral load, full blood count and lymphocyte subsets determined as part of their routine medical care at baseline, 12 and 24 weeks. In addition, patients donated blood for flow cytometry analysis at baseline and the routine follow-up visits at 4, 8, 12 and 24 week visits. Plasma was stored at -20°C.

The study was approved by the Gambian Government/ MRC Ethics Committee.

7.2.4 Enumeration of Tregs using FACS staining of whole blood

Fresh whole blood (150 µl) was stained within 6 hours using conjugated monoclonal antibodies to CD25-FITC, CD4-PerCP, CD3-PE (Becton Dickinson, USA) and FOXP3-APC (Clone: PCH101, Ebiosciences, USA) as described in Chapter 3, Section 3.4.

7.2.5 CD4 T cell counts and viral load measurements:

Enumeration of CD4 levels (CD4% of total CD3 T cells) and viral loads were determined at baseline and follow-up visits as described in Chapter 3.

7.2.6 Measurement of plasma cytokine levels:

Plasma levels of IL-2, IL-6, IL-10, IL-12, IL-13, IL-17, TNF- α , MIP-1 β and IFN- γ were measured at baseline using a single Bioplex assay (Bio-Rad, USA) on undiluted plasma as described in Chapter 3.

7.2.7 Analysis

Patients were classified into IRIS and non-IRIS groups. Patients who interrupted ART > 1 week, or stopped ART before 12 weeks, were excluded from the immunological analysis.

Longitudinal proportions and absolute levels of Tregs were analyzed using generalized estimating equations (equal correlation structure) adjusting for age, sex, ethnicity and viral loads. Cross-sectional analyses of baseline response were compared between the IRIS and non-IRIS groups adjusted for the same possible confounders as mentioned above. Tests with a p-value < 0.05 were considered statistically significant. All analyses were performed in Stata (version 11) and figures were drawn using Prism (version 5).

7.3 Results

7.3.1 Incidence of IRIS in the study cohort

Of 80 patients enrolled after informed consent, 71 (89%) completed more than 12 weeks of ART without an interruption of greater than one week. One patient was found to have CD4 count >800 cells/ μ l twice on repeat baseline testing, and was subsequently taken off ART. Four patients were lost to follow-up before 12 weeks, and 2 patients interrupted ART for 1 month. Three patients died, of whom two had interrupted ART for more than four weeks prior to death without IRIS symptoms recorded at the last visit. One patient, in whom IRIS could not be excluded, died after 8 weeks on ART.

Of the 71 patients included in the analysis, 21 (30%) developed symptoms consistent with IRIS using the criteria set out by Haddow *et al* (2009). Fifteen (71%) of these patients were classified as cases of unmasking IRIS.

IRIS patients typically had highly advanced disease and commenced therapy with a significantly lower nadir CD4 count than non-IRIS patients (60 versus 120 cells/ μ l, $p=0.03$). Two of the 9 HIV-2 infected subjects and one of the 4 dually infected subjects developed IRIS.

However, the IRIS group also showed a significantly greater increase in absolute CD4 T cells levels compared with non-IRIS group six months after commencement of antiretroviral therapy (150 versus 100 cells/ μ l, $p=0.04$). All subjects showed excellent responses to treatment and 92% of patients had an undetectable viral load at the end of the follow-up period (Table 7.1 and Figure 7.1).

Table 7.1. Patient characteristics

	All	IRIS	Non-IRIS	P value
N (%)	71	20(28%)	51(72%)	
Male	25 (35%)	5 (24%)	20 (39%)	
Age in years (median)	41	39	43	ns
CD4 nadir (median) Cells/ μ l	100 (30-160)	60 (20-130)	120 (45-180)	0.03
CD4 increase in 6 mo (median) Cells/ μ l	120 (70-205)	150 (120-330)	100 (50-180)	0.01
HIV type				
1	58 (82%)	17(85%)	41(80%)	ns
2	9 (13%)	2 (10%)	7(14%)	ns
dual	4 (6%)	1 (5%)	3(6%)	ns
VL < 100 copies/ml	65 (92%)	18(90%)	47(92%)	
Ethnicity				
Mandinka	28 (39%)	7(30%)	21(41%)	ns
Wolof	9 (13%)	2(10%)	7(14%)	ns
Fula	8 (11%)	3(10%)	5(10%)	ns
Jola	8 (11%)	3(10%)	5(10%)	ns
Other	18 (25%)	5 (20%)	13(25%)	ns
ART 24wk	61 (86%)	19 (95%)	42(69%)	ns

IRIS= Immune reconstitution inflammatory syndrome

ADE= Adverse drug effects

VL= Viral load, <100= undetectable within first 9 months of ART

ART= Antiretroviral therapy

ART 24wk= No interruption in ART use >3 days over 24 weeks

ns= not significantly different, $p>0.05$

The majority of patients that developed IRIS showed symptoms within the first month after initiation of ART (16 of 20 (80%)). The median duration of IRIS symptoms was 49 (IQR: 28-91) days. Pathogens were rarely identified and the lung and skin or mucosal surfaces were involved in most cases (Table 7.2). Eight of the twenty patients who developed IRIS had developed TB, for which they were treated before commencing ART. Of note there were nine HIV-2 infected subjects that were included in this study and two of these developed IRIS.

Age (yrs)	Sex	HIV	CD4 Nadir (cells/ μ l)	IRIS (week)	IRIS Duration (days)	Predominant Organ	nadir VL copies/ml)	CD4 increase (cells/ μ l)
40	F	1	50	2	91	Skin	<100	50
38	F	2	130	3	110	Pulmonary	<100	150
29	F	1	220	2	42	Pulmonary	<100	330
21	F	1	70	2	66	Renal/Pulmonary	<100	390
38	F	1	20	12	296	Skin	985	110
33	F	1	100	4	14	Pulmonary	<100	120
35	M	1	10	1	91	Pulmonary	<100	130
30	F	11	130	1	32	Skin	<100	150
40	F	1	20	4	49	Skin	<100	210
31	F	1	90	4	40	Pulmonary	<100	240
44	F	1	130	8	88	Pulmonary	<100	280
35	F	1	100	2	70	Skin	<100	300
26	F	2	10	4	49	Skin	<100	330
47	M	11	60	2	28	Pulmonary	<100	350
39	F	1	10	2	14	Pulmonary	<100	410
33	F	1	80	3	30	Skin	5014	580
46	F	1	50	3	67	Pulmonary	<100	60
44	M	1	140	2	13	Skin	<100	80
45	M	1	10	12	84	Lymph	<100	90
41	M	1&2	30	7	35	Lymph	<100	90

Table 7.2: Features of IRIS patients.

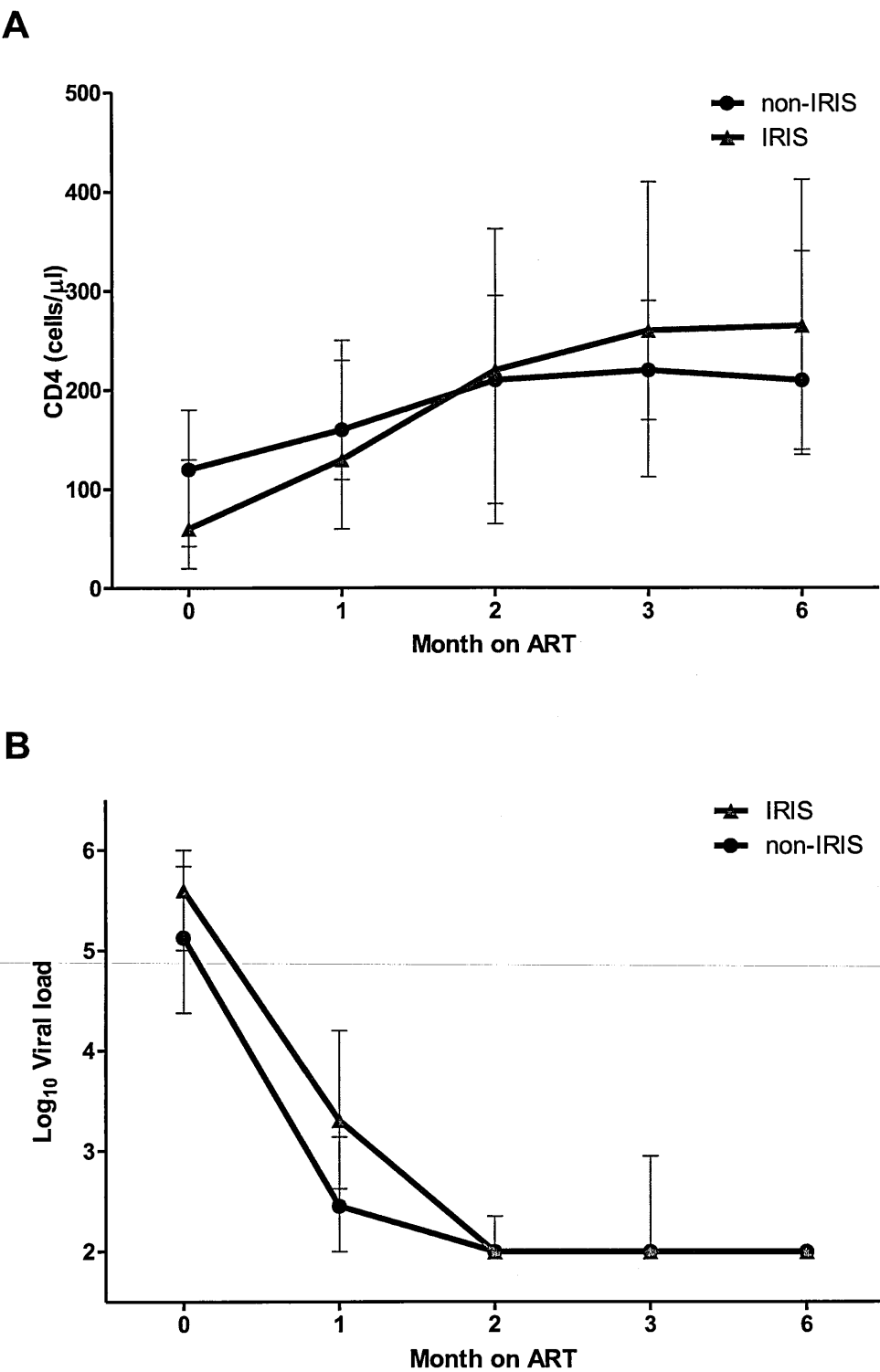


Figure 7.1: Median CD4 levels (A) and viral loads (B) at different times after initiation of ART in IRIS and non-IRIS group. The vertical bars represent the interquartile range.

7.3.2 T regulatory cell reconstitution in patients with and without IRIS

In Chapter 5 (Figure 5.2C) it was shown that the levels of CD4⁺FOXP3⁺ and CD4⁺CD127^{lo/-} FOXP3⁺ cells were highly correlated in both HIV-1 and HIV-2 patients. As an automatic processing tool was available to determine CD4⁺FOXP3⁺ events we chose to define Tregs as CD4 T cells co-expressing the transcription factor FOXP3. Using this tool it was possible to objectively quantify Treg levels in all patient samples. The output from the algorithm was consistent and comparable to manual analysis (Jeffries *et al.*, 2008). The gating strategy employed to enumerate Tregs as a percentage of total CD4 T cells is shown in Figure 7.2.

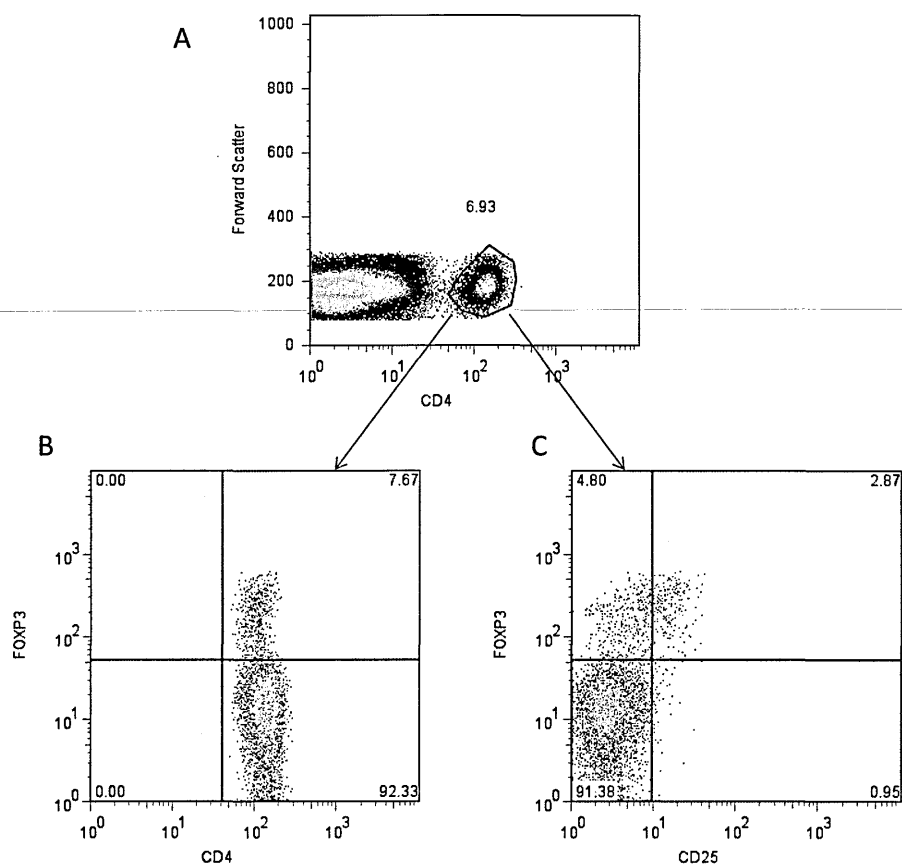


Figure 7.2: Gating strategy to determine the levels of Tregs. A) CD4 versus forward scatter B) CD4 versus FOXP3 and C) CD25 versus FOXP3

No significant difference was observed in percentage Treg levels between the IRIS and non-IRIS groups before initiation of ART (Month 0). Tregs expressed as a percentage of total CD4 T cells declined after ART initiation, a trend which was evident in both patient groups (Figure 7.3A). Indeed, comparison of Treg levels at baseline (median: 15.5%) and six months (median: 6.6%) after commencement of ART showed a significant reduction in all patients ($p= 0.002$). Similarly, there was no significant difference when comparing levels of absolute Tregs at baseline or six months after ART in any of the groups (Figure 7.4B). These trends were observed regardless of the definition of Tregs ($CD4^+FOXP3^+$ versus $CD4^+CD25^+FOXP3^+$).

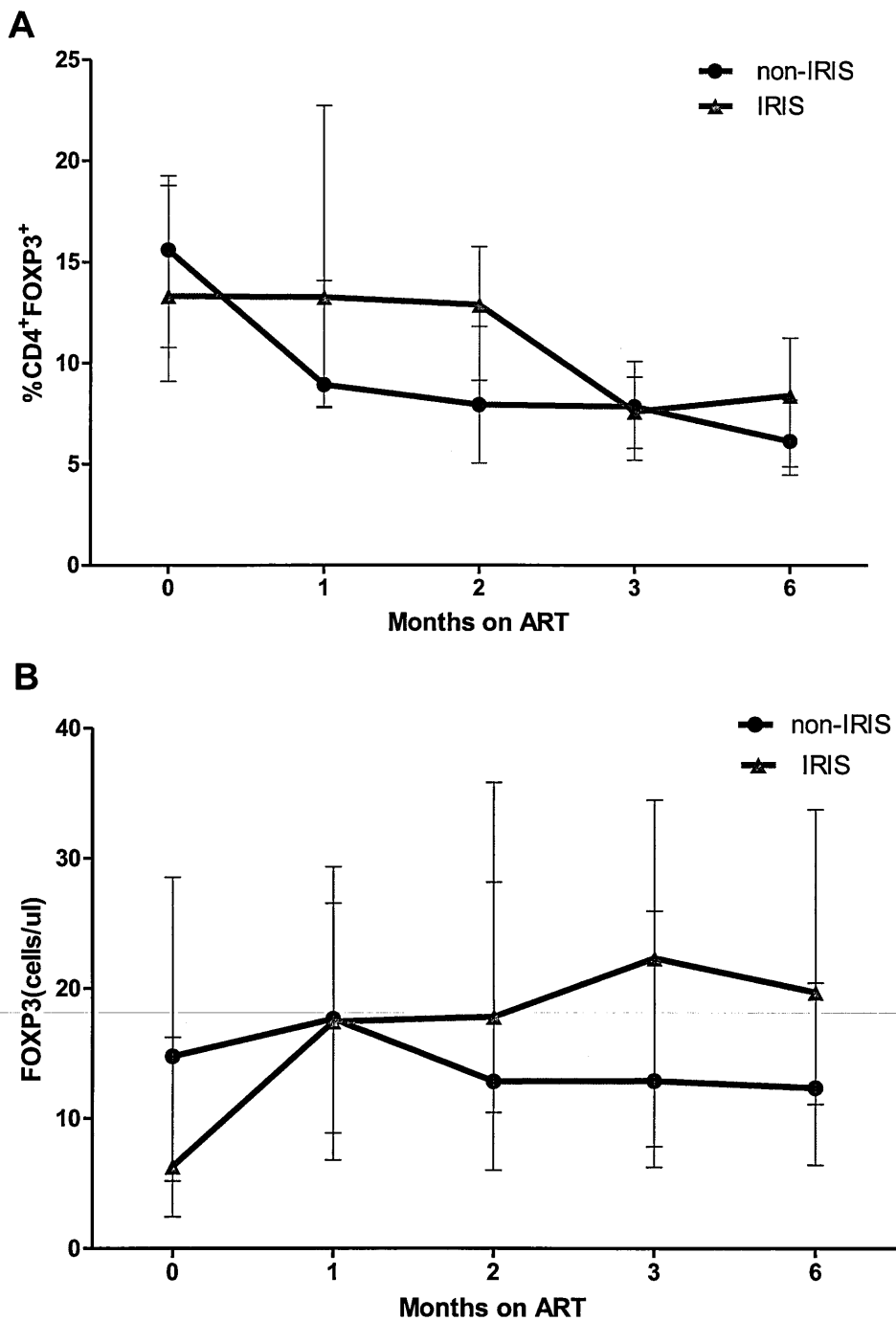


Figure 7.3: Median and IQR range of Tregs at different times after start of ART expressed as a A) Percentage of total CD4⁺ T cells or B) Absolute numbers of Tregs (cells/μl). The vertical bars represent the interquartile range at each time point.

7.3.4 Baseline cytokines and chemokines do not predict IRIS

Plasma cytokine levels were measured at baseline to determine whether these could be used to predict the onset of IRIS. The levels of IL-12, IL-13, and IL-17 were below the limit of the assay in all patients tested. Plasma levels of IL-2, IL-6, IFN- γ MIP-1 β , IL-10 and TNF- α were comparable in both groups (Table 7.3). There was no significant correlation observed between the levels of CD4⁺FOXP3⁺ cells and any of the cytokines measured.

Category	Cytokine (pg/ml)					
	IL-2	IL-6	IFN- γ	MIP-1 β	TNF- α	IL-10
Non-IRIS	0.765 (0-12.27)	7.4 (3.16-10.83)	10.65 (4.57-34.66)	73.65 (54.11-124.91)	0 (0-1.67)	2.84 (1.21-4.22)
IRIS	4.86 (0-28.74)	11.16 (3.84-25.47)	27.11 (4.57-243.78)	72.52 (56.49-98.21)	2.1 (0-4.17)	4.38 (1.45-9.98)

Table 7.3: Cytokine profile of patients before commencement of therapy. The median and interquartile ranges are shown.

7.4 Discussion

The definition of IRIS remains elusive, both as a clinical phenotype with imprecise case description and as an immunological phenomenon which occurs during the massive overhaul of the immune system as people start ART. Several case definitions have been proposed for TB-IRIS (Meintjes *et al.*, 2008) and all-cause IRIS. The most recent all-cause IRIS case definition was used in this study which has been validated before (Haddow *et al.*, 2009).

The incidence of IRIS (28%) was comparable with similar studies in South Africa and Mozambique and lower than that reported in Senegal (Letang *et al.*, 2011, Muller *et al.*, 2010). In our study IRIS patients had a significantly lower nadir CD4 T cell count and showed a greater recovery of CD4 T cell count compared with the non-IRIS group. These results re-affirm that a low CD4 T cell count at the time of initiating ART is a risk factor for the development of IRIS (Letang *et al.*, 2011, Grant *et al.*, 2011, Ratnam *et al.*, 2006, Jevtovic *et al.*, 2005)

The majority of IRIS cases developed within the first month after starting on ART. In addition the IRIS patients showed a higher recovery of CD4 T cells compared to the non-IRIS group. These findings emphasize the need for commencing ART early and for close monitoring of HIV infected patients after the start of ART. Unique to this study were 9 patients infected with HIV-2; two of these patients developed IRIS. This emphasizes the point that in HIV-2 infected individuals who progress to AIDS, the clinical course after treatment may mirror that of HIV-1 infected individuals.

Understanding the immunopathogenesis of IRIS remains a challenge. Higher levels of immune activation prior to commencement of ART have been identified as a risk factor for development of IRIS. Hence, it was hypothesized that there was a difference of Treg

dynamics in patients commencing ART that could distinguish IRIS from non-IRIS cases. Although we noted lower levels of absolute Tregs at baseline in those patients who would develop IRIS the differences were not significant and the percentage of Tregs were similar in both groups of patients. The proportion of Tregs in all patients declined steeply after the initiation of ART, which is consistent with the results of a recently published study (Montes *et al.*, 2010).

There were no clear differences in the trend of percentage or absolute Tregs between IRIS and non-IRIS groups. However the data was highly variable thus it cannot be ruled out that Treg dynamics within the first month after initiation of ART may influence the development of IRIS. Future studies would require more intensive sampling during the first few weeks after initiation of ART.

The results are consistent with a previous study comparing Treg levels in TB patients commencing ART, which similarly showed no significant differences in the percentage of Tregs at the time of IRIS presentation versus non-IRIS cases two weeks post ART treatment (Meintjes *et al.*, 2008). While neither the TB-IRIS study nor our study included functional assays, a cross-sectional study on 8 patients with *M. avium* related IRIS identified high numbers of Tregs (Seddiki *et al* 2009). However these Tregs were unable to suppress the secretion of pro-inflammatory cytokines IFN- γ , TNF- α and IL-6 *in vitro*, suggesting that a defect of regulatory T cells may indeed contribute to IRIS. It is therefore plausible that despite high frequencies of Tregs, IRIS may result from inadequate suppressive function of these cells. Further studies that can quantitate Treg function at ART initiation and prior to development of IRIS may clarify whether Treg dysfunction rather than changes in numbers indeed contributes to IRIS.

Higher levels of pro-inflammatory cytokines before resumption of ART have been linked to a greater risk of developing IRIS (Reviewed by Sereti *et al.*, 2010). Even though there were higher median levels of IL-6 and IFN- γ in the IRIS group in our study compared to the non-IRIS group at baseline, these differences were small and did not predict the development of IRIS.

Heterogeneity within the diagnostic category of IRIS, including unmasking of active infection, response to residual antigens, non-antigen driven immune responses, and involvement of different organ systems, further limits the interpretation of our findings as these could represent substantially different immunologic pathways. Although research on IRIS is limited by the mutually reinforcing challenges of inadequate clinical case definitions and a lack of biomarkers related to its pathogenesis, recent data focused on *M. tuberculosis* related IRIS suggest that this syndrome depends on interactions between the residual components of *M. tuberculosis*, which serve as stimuli for innate immune- or specific T cell responses, the partially reconstituted cellular immune response, and incomplete regulation of the reconstituted immune system. IRIS can occur within days of commencement of ART, prior to witnessed increases in CD4 counts, which suggests a role for other components of the immune system. The strong granulomatous response commonly seen in IRIS (Lawn *et al.*, 2009) has led to the hypothesis that the granulomas result from macrophages resuming normal function with the decrease in viral load (Van den Berg *et al.*, 2006). This is supported by findings that *in vitro* gp120 stimulation of monocyte-derived macrophages is sufficient to induce profound changes in macrophage gene expression patterns (Cicala *et al.*, 2002).

In summary the results of this study show that neither Treg frequencies nor cytokine levels predict the onset of IRIS. Further investigation is required to assess Treg function to

determine whether these cells influence innate and adaptive responses that contribute to the development of IRIS.

Chapter 8: Final Discussion

HIV infection remains a significant cause of morbidity and mortality around the world despite concerted efforts to curtail its spread. The introduction of ART to AIDS endemic areas has reduced the mortality associated with HIV infections but it is estimated that for every one person started on ART therapy another five new infections occur (UNAIDS Global Report 2010). This highlights the need for an effective vaccine that prevents the spread of HIV infection. Recent positive developments from the RV144 vaccine trial have encouraged researchers and this suggests that it is possible to develop an efficacious vaccine (de Souza *et al.*, 2009). However, a major roadblock in developing a vaccine is an incomplete understanding of the immunology of HIV infection. Our knowledge of the correlates of immune protection in HIV-1 remains incomplete due to the scarcity of patients that control the replication of their virus who are available for study. The studies of HIV-2 infection have the potential to provide valuable insight into mechanisms of non-progression, since unlike HIV-1, the majority of HIV-2 infected patients efficiently control viral replication and maintain the integrity of their immune system (Berry *et al.*, 2002, Leligidowicz *et al.*, 2007, Nuvor *et al.*, 2006, Duvall *et al.*, 2006). Both infections are characterized by chronic immune activation (Michel *et al.*, 2002, Leligidowicz *et al.*, 2008), high levels of which can accurately predict progression to AIDS (Jaffar *et al.* 2005). Hence, understanding the factors that contribute to control of immune activation would assist in the development of an efficacious vaccine.

T regulatory cells are potent suppressors of cellular activation which suggests that they may limit the ability of HIV virus to infect and replicate since they preferentially target activated T cells. However, the suppressive activity of Tregs has also been shown to diminish HIV specific immune responses (Andersson *et al.*, 2005, Aandahl *et al.* 2004,

Kinter et. al 2004, 2007, Weiss *et al.*, 2004). Hence, there is continued debate on whether the Treg activity is beneficial or detrimental in HIV disease progression. While there have been many studies describing these cells in HIV-1 infections, there has been only one previous study which attempted to quantify and compare Tregs in HIV-1 and HIV-2 infection (Foxall *et al* 2008). This study used FOXP3 mRNA as a surrogate marker for these cells. In my work I attempted to define and compare the role of Tregs in HIV-1 and HIV-2 infected individuals by studying their phenotype, frequencies and functional characteristics.

A major caveat in the study of Tregs in HIV infection has been the lack of an appropriate cellular phenotype to identify these cells. Other studies used FOXP3 mRNA levels as a surrogate for total Treg levels but in most cases these failed to correlate them with cell frequencies (Kinter *et al.*, 2005, Yamamoto *et al.*, 2008). In Chapter 4 of this thesis, it was shown that CD4 T cells that expressed FOXP3 also exhibited CD127^{lo/-} co-expression and that these cells were heterogeneous in their expression of CD25. Furthermore, CD4⁺FOXP3⁺ cells were predominantly at an early differentiation stage (as determined by co-expression of CD45RO and CD27) but there was also a population of FOXP3 cells with a naïve phenotype (CD45RO⁻CD27⁺) in healthy donors and HIV infected subjects. This proportion of naïve Tregs was similar in HIV-1, HIV-2 and healthy individuals, which suggests that it is a true population and not the result of activation induced FOXP3 expression as suggested by others.

I also attempted to assess whether the transcript levels of FOXP3 measured by real time PCR and number of Tregs (measured by flow cytometry) were correlated in HIV infected patients. The results clearly showed that the measurement from the two methods did not

correlate and suggests that new methodologies used to enumerate Tregs should be carefully evaluated.

In some cases HIV-2 infection can lead to disease progression similar to that observed in HIV-1 infection. The second study performed measured Tregs as a percentage of total CD4 T cells or as absolute numbers to determine their relationship with disease progression. This comparison clearly demonstrated that HIV-2 asymptomatic patients with high CD4 counts had significantly lower levels compared to HIV-1 patients matched by CD4 T cell counts or HIV-2 patients who had evidence of disease progression. The asymptomatic patients had the lowest levels of immune activation and median viral loads. The findings suggest that low percentage of Treg levels are a feature of non-progression and such HIV-2 patients had been infected for longer than HIV-1 patients and HIV-2 progressors. Furthermore, Treg levels positively correlated with immune activation and viral loads in HIV-2 infections only. These results suggest that increasing activation as a result of viral replication in HIV-2 may be countered by an increased induction of Tregs.

The lack of this relationship in HIV-1 patients suggests that the balance between effector and regulatory mechanisms is impaired. The majority of HIV-1 infections are characterized by higher levels of viral replication compared to HIV-2. This results in chronic stimulation of the immune system and increased levels of immune activation. This may account for the higher levels of Tregs observed in asymptomatic HIV-1 infection and would negatively influence HIV specific immune responses. In contrast, the lower levels of Tregs in HIV-2 infection may be due to lower immune activation as a result of reduced viral replication.

The immune function in HIV-2 asymptomatic patients has been shown to be better preserved compared to that in HIV-1 infections. To explore the balance between immune

activation and HIV-2 specific immune responses, patients with low Tregs were selected for further studies in parallel with HIV-1 patients. The comparison of CD4 and CD8 HIV specific immune responses revealed superior magnitude and polyfunctionality in HIV-2 patients compared to HIV-1. Furthermore, the levels of activation in the CD4 and CD8 T cell compartments were also significantly lower in HIV-2 group. This suggests that long term non-progression is associated with low levels of Tregs, preserved and polyfunctional T cell responses and low activation levels. These results are further strengthened by similar studies conducted on HIV-1 elite controllers which have been show them to have low levels of immune activation, low Treg numbers and a high level of HIV specific polyfunctional T cell responses (Schulze zur Weisch *et al* 2011; Owen *et al* 2010).

IRIS develops in some patients commencing ART therapy. It was hypothesized that delayed reconstitution of Tregs may be a factor in the development of IRIS. In agreement with previous reports, IRIS patients had lower CD4 counts at their nadir and a better reconstitution of CD4 cells than the non-IRIS group. Surprisingly though, there was no clear differences observed in the increase during treatment of Tregs in patients who developed IRIS and those who did not. Furthermore, measurement of baseline cytokine levels or Tregs did not discriminate the two groups. IRIS encompasses a broad range of clinical symptoms and the heterogeneity in the IRIS classification may have contributed to the negative finding. While it was not possible to explore functional characteristics of the cells that contribute to IRIS or Tregs, further studies are warranted to decipher which correlates will identify patients at risk of developing IRIS.

Understanding the factors associated with non-progression during HIV infection are essential for designing a viable vaccine. HIV-2 presents a physiologically relevant human model to assist in this endeavour. This study has shown that HIV-2 non-progression is

associated with lower levels of Tregs, preserved immune function and low immune activation. Further studies are warranted to compare the function of Tregs between HIV-2 progressors and non-progressors. Furthermore, better correlates of IRIS are required so as to be able to identify patients at risk before initiation of ART.

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APPENDIX

Clinical guidelines for defining IRIS cases as outlined by Haddow *et al* 2009.

Paradoxical IRIS

Clinical criteria

1. Temporal relationship: ART initiation must precede clinical deterioration
2. One of the following:
 - a. Worsening of an infectious or inflammatory condition that was recognized and ongoing at the time of ART initiation, following a clinical response to appropriate treatment
 - b. Deterioration with atypical or exaggerated clinical, histological, or radiologic findings in terms of severity, character of inflammatory response, rapidity of onset, or localization
 - c. Recurrence of an episodic infectious or inflammatory condition, worse than episodes within 1 year preceding ART in terms of frequency, severity, or response to therapy

Exclusion of other causes

Worsening not explained by

1. Expected clinical course of underlying condition, given current therapy and the susceptibility profile of the organism
2. Drug toxicity
3. Other infection or inflammatory condition
4. Withdrawal of previously effective therapy
5. Failure of antiretroviral treatment: presumptive, based on either non-adherence or resistance to ART, or confirmed, based on VL assay if available.

Unmasking IRIS

Clinical criteria

1. Temporal relationship: ART initiation must precede clinical deterioration
2. New onset of symptoms of an infectious or inflammatory condition after initiation of ART
3. Consistent with the presence of preexisting causative pathogen or antigen at the time of starting ART
4. Either of the following:
 - a. Onset within 3 months after initiating ART
 - b. Atypical or exaggerated clinical, histological, or radiological findings in terms of severity, character of inflammatory response, rapidity of onset, or localization

Exclusion of other causes

Event not explained by

1. Expected clinical course of another condition
-
2. Drug toxicity
 3. Newly acquired infection, based on clinical history or other evidence
 4. Failure of antiretroviral treatment: presumptive, based on either non-adherence or resistance to ART, or confirmed, based on VL assay if available

The Gambia Government / MRC Laboratories Joint

ETHICS COMMITTEE

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Mr Irfan Zaidi
MRC Laboratories
Fajara

30th March 2006

Dear Mr Zaidi

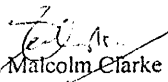
Re: Project 1035v3. 10th March 2006. The role of Regulatory T cells in the pathogenesis of HIV-2 infection

Thank you for submitting the above project, which was reviewed by the Joint Gambia Government/MRC Ethics Committee at its meeting of 24th March 2006.

The project and the information sheet and consent form relating to the Fajara cohort was approved. It is understood that the information sheet and consent forms relating to the Caio cohort will be submitted in due course before beginning that arm of the study.

With best wishes

Yours sincerely



Mr. Malcolm Clarke
Chairman

Gambia Government/MRC Joint Ethics Committee

cc: Professor Sarah Rowland-Jones

The Gambia Government / MRC Laboratories Joint Ethics Committee:

*Mr Malcolm Clarke, Chairman
Mrs Kathy Hill, Secretary
Mrs Norwoh Ali, 2nd Secretary
Dr Ousman Nyan, Scientific Advisor
Mr Dawda Jagne
Mrs Bertha Mboge*

*Dr Tumani Corrah
Professor Hilton Whittle
Dr Stephen Howie
Dr Mariatou Jallow
Dr. Lamin Sidibeh
Mr. Malamin Sonko*

T regulatory Cell Study Patient Consent Form

I have read, or had read to me, the information sheet for this study. I understand what it says, and what being in the T regulatory cell study means for me. I had a chance to ask questions about the study, and had them answered.

I understand that the information regarding me, including my medical status and the results of studies done on blood samples, will remain confidential.

I understand that part of the blood samples that I provide will be stored for possible future tests.

I understand that if I get sick during the study period, I can go to the clinic where study staff is providing care, and be examined and treated for free.

I understand that I am free to take part in the study or refuse, and that I can withdraw from the study at any time, and without giving any reason. Deciding not to take part or to withdraw from the study will not affect the care that I am normally entitled to.

Please check one of the following:

I am in other research studies.

☐ I am not in other research studies.

Signature or thumb print of volunteer: _____

Date: | | | / | | | / | | |

[illegible]

This form has been read by / I have read the above to _____
(write name of volunteer)

in a language that he/she understands. I believe that he/she has understood what I explained and that he/she has freely agreed to take part in the study.

Signature of field worker: _____

Name of field worker: _____

Date: | | | / | | / | | | |

T Regulatory Cell Study Information for Participants

What is the problem with HIV? HIV is a chronic infection, which in some cases badly damages the immune system. At the moment there is no cure for the disease but we can slow down progress of the disease.

What are T regulatory cells? T regulatory cells are important in controlling the response of your body to infection. This ensures an appropriate response such that any inflammation is reduced

What is this study about? The object of this study is to understand the role of T regulatory cells in HIV infected individuals.

Why are we asking you to participate? As a member of the HIV cohort we have been monitoring your progress. We would like to invite you to join the study so we can see whether T regulatory cells are helpful in keeping you healthy.

What happens if you agree to participate? Your participation in this study is entirely voluntary. Being in this study is not likely to help or harm you in any significant way. You will not be paid to participate in this research study. We are not asking to bleed you in addition to what you have consented for when joining the cohort. A small portion of the 15ml (3 teaspoons) of blood taken from you will be used for routine monitoring of your immune system (CD4 count and viral load). We will use the remainder of the blood to measure the number of T regulatory cells and other tests to check they are functional. If there is any remaining blood it will be stored for later, as yet unspecified, scientific tests focused on immunity to HIV.

Questions? Please feel free to ask any questions you want at any time during the study. All personal data collected for this study will be treated as confidential information. Information (other than the name or other identifying information) gathered in the course of medical care at MRC may be used in the development or answering of research questions related to medical care, and to inform public health officials and policy makers about health-related conditions in the Gambia.

You are free to leave the study at any time you so wish. This will not affect your access to normal medical care. You should not feel obligated to agree to participate. Your questions should be answered clearly and to your satisfaction. If you are willing to help us with this study, we would like you to sign the consent form.
